PRODUCTION OF TRANSGENIC CITRUS PLANTS EXPRESSING THE CITRUS TRISTEZA VIRUS COAT PROTEIN GENE

By

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To my parents Jorge Gutierrez Samperio and Maria Elena Espinosa Perea, my sisters Adriana and Laura Elena and brother Jorge Antonio.

To my husband Gustavo Mora Aguilera who is the world to me.

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PRODUCTION OF TRANSGENIC CITRUS PLANTS EXPRESSING THE CITRUS TRISTEZA VIRUS COAT PROTEIN GENE

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Chair: Dr. Gloria A. Moore
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Citrus tristeza virus (CTV) is distributed worldwide and is the most devastating viral citrus disease. The objectives of this study were to obtain transgenic citrus plants and to evaluate the cross protecting ability of transgenic sour orange citrus plants expressing the coat protein of the CTV when challenged with the severe strain T-36. It has been demonstrated that transgenic plants expressing a plant virus coat protein (CP) can be resistant to infection by the homologous virus. Powell-Abel and collaborators were the first to demonstrate that transgenic tobacco expressing the coat protein gene of tobacco mosaic virus (TMV) was resistant to TMV infection. Many other reports have since shown that this phenomenon, referred to as pathogen-derived resistance by some and as coat protein-mediated protection (CPMP) by others, is broadly applicable to different groups of plant viruses. Importantly, this phenomenon has obvious applications to plant protection because it provides a direct approach for incorporating resistance into commercially acceptable cultivars or breeding lines. We have produced transgenic citrus plants that express the CP gene from CTV strain T36 using an *Agrobacterium*-mediated method. The effects of *Agrobacterium* strain/vector plasmid combinations, hormone concentration, and citrus genotype on the frequency of transgenic plant production were investigated. The greatest improvement in transformation frequency [β-glucuronidase positive (GUS+ shoots)] was obtained with vector plasmid pGA482GG in combination with *A. tumefaciens* strain EHA101. Increased concentration of cytokinin 6-benzyladenine (BA) induced higher transformation frequencies as a result of the large number of shoots that regenerated. However, there appeared to be a carry-over effect of BA in regeneration medium on rooting frequency: the more BA in the medium, the fewer of the produced shoots rooted. Transgenic sour orange plants (*C.aurantium* L.) and Carrizo citrange [*C. sinensis* (L.) Osb. x *Poncirus trifoliata* (L.) Raf.] expressing the CP were confirmed by Protein blot analysis and transgenic key lime plants (*C. aurantifolia* [Christm.] Swing.) were PCR positive for the presence of CTV-CP. Single and double T-DNA insertions were detected by DNA blot analysis of transformed plants. Transgenic sour orange plants have been propagated and grafted in various configurations with susceptible material, and were challenged with a severe strain (T-36) of the virus under greenhouse conditions. Coat protein-mediated protection was evaluated by detecting the replication or the lack of replication of CTV isolates in the inoculated plants. Enzyme-linked immunosorbent assays (ELISA) were conducted to determine presence/absence of the virus.

CHAPTER 1
INTRODUCTION

The ability to transfer a single gene into a plant cell and subsequentlly produce an entire plant from the transformed cell is well established. Genetic transformation is a very attractive procedure to handle crop improvement in plants that are highly heterozygous, have an extended sexual generation and are propagated mainly vegetatively. This is the case of most fruit trees such *Citrus* cultivars. A major advantage of using genetic transformation, rather than conventional cross-breeding, in fruit trees, is that a specific advantageous trait may be added to a given cultivar or rootstock genome while avoiding burdens of sexual recombination and involvement of deleterious characteristics. The more commonly used techniques to achieve plant transformation include *Agrobacterium tumefaciens* T-DNA-mediated transfer, direct DNA transfer into protoplasts, and microprojectile bombardment. Most methods of gene transfer rely on *in vitro* plant regeneration.

Furthermore, genetic transformation of plant genomes with viral coat protein genes has been shown to confer a type of cross protection to the previously susceptible plants. Transgenic tobacco plants engineered with the Tobacco Mosaic Virus (TMV) coat protein exhibited delayed disease symptoms and 10 to 60 percent of the transgenic plants failed to develop symptoms for a period of time (Powell-Abel *et al.* 1986). Many other reports have since shown that this phenomenon, referred to as pathogen-derived resistance (Lindbo and Dougherty 1992a) by some and as coat protein-mediated protection (CPMP) by others (Gonsalves *et al.* 1992, 1994), is broadly applicable to different groups of plant viruses. Importantly, this phenomenon has obvious applications to plant protection because it provides a direct approach for incorporating resistance into commercially acceptable cultivars or breeding lines. Recent reports have expanded this pathogen-derived resistance to other commercially important crops. Transgenic tomato (Nelson *et al.* 1988), alfalfa (Hill *et al.* 1991), potato (Kawchuk *et al.* 1990, Mackenzie *et al.* 1991) muskmelon (Gonsalves *et al.* 1994) and cucumber (Gonsalves *et al.* 1992), expressing the coat protein gene of either cucumber mosaic (CMV), alfalfa mosaic (AMV), potato virus S (PVS), potato leafroll (PLRV), cucumber mosaic virus-white leaf strain (CMV-WL) or cucumber mosaic-C strain (CMV-C) viruses have been developed and shown to be resistant to these specific viruses.

Ultimately, the value of the newly acquired resistance must be translated into field performance of the transgenic crops. To this end, a few studies have shown that transgenic potatoes, tomatoes and cucumbers are resistant to Potato virus Y (PVY) and Potato virus X (PVX) (Kaniewski *et al.* 1990), TMV (Nelson *et al.* 1988), and CMV-C (Gonsalves *et al.* 1992) under field conditions following mechanical inoculation of the challenge virus.

Citrus meets two major requirements for obtaining transgenic plants. First, tissue-culture procedures have been established (Murashige and Tucker 1969, Spiegel-Roy and Vardi 1984, Litz *et al.* 1985, Moore 1985; 1986, Beloualy 1991). Second, *Citrus* cells are susceptible to infection by *A. tumefaciens* (Moore *et al.* 1989; 1992, Kaneyoshi *et al.* 1994, Peña *et al.* 1995a, 1995b). Furthermore, a cDNA to the citrus tristeza virus (CTV) coat protein gene (CTV-CP) was generated that can be used to obtain transformed citrus plants (Sekiya 1990). This cDNA may confer the novel type of resistance seen in tobacco plants and other crops to suitable citrus cultivars.

The objectives of this study were to obtain transformed citrus plants containing the citrus tristeza virus coat protein gene (CTV-CP) using *A. tumefaciens* and to assess under greenhouse conditions the cross protecting ability of transgenic sour orange citrus plants expressing the coat protein of the CTV when challenged with severe strain T-36 or CTV isolate T-64. Factors that affect the frequency of transformation in citrus were also addressed.

CHAPTER 2
LITERATURE REVIEW

Plant Transformation

Plant transformation has been described as transfer of one or two defined genes followed by integration into the host genome and expression (Lindsey 1992). The stable introduction of foreign genes into plants represents a significant development in modern plant breeding. In general terms, the overall aims of breeders are 1) an improved product yield and quality; 2) an improved ease of harvest of the crop; 3) an increased resistance to disease and various environmental stresses such as drought, flooding, extremes of temperature, and high soil content of salt and heavy metals; and 4) improved post-harvest properties such as ease of processing and the maintenance of quality during storage. There are a number of problems that are difficult to overcome by conventional methodologies, which are a consequence of the genetic and reproductive characteristics of the plants themselves. These include genetic linkage, polygenic traits, mutations, time scales and size of the available gene pool. Genetic engineering methods complement plant breeding efforts by increasing the diversity of genes and germplasm available for incorporation into crops and by shortening the time required for the production of new varieties.

During the 1980s a number of reports showed that plants were amenable to genetic engineering. Transformation was confirmed by the presence of foreign DNA sequences in both primary transformants and their progeny and by an antibiotic resistance phenotype conferred by a chimeric neomycin phosphotransferase gene (DeBlock *et al.* 1984, Gasser and Fraley 1989). Since then a variety of techniques have been used to stably introduce foreign genes into plant chromosomal DNA (Potrykus 1991). These techniques can be described either as exploiting vector systems or as being vector-free. By far the most widely used vector-based transformation system makes use of the natural gene-transfer properties of the soil-borne pathogen *Agrobacterium tumefaciens* (Klee *et al.* 1987).

The host range of *Agrobacterium* is limited, which has lead to the development of alternative, vector-free or direct gene transfer systems for plant transformation. A common feature of these is that the objective is to overcome the barrier to the free movement of DNA which the plasma membrane represents. Direct gene transfer systems are characterized by the transfer of naked DNA molecules into, most commonly, protoplasts, through facilitation of DNA uptake by calcium phosphate precipitation, polyethylene glycol treatment, electroporation, microinjection or combinations of these treatments, followed by the regeneration of stably transformed callus and plants under antibiotic selection conditions. Another direct gene transfer system is the bombardment of cells or tissues with metal microprojectiles (e.g. tungsten or gold beads) which are coated with DNA, known as particle gun or high-velocity microprojectile technology (Klein *et al.* 1987). With this system, the microprojectile particles are accelerated at high speed into cells or even into intact plant tissue.

Agrobacterium -Mediated Transformation

Derivatives of the plant pathogen *Agrobacterium tumefaciens* have proved to be efficient, highly versatile vehicles for the introduction of genes into plant cells. *A. tumefaciens* is the etiological agent of crown gall disease and produces tumorous galls when inoculated on some species. The utility of this bacterium as a gene transfer system was first recognized when it was demonstrated that the crown galls were actually produced as a result of the transfer and integration of genes from the bacterium into the genome of plant cells (Chilton *et al.* 1977). Virulent strains of *A. tumefaciens* have a large (150 to 250 Kb) plasmid called the Ti (tumor inducing) plasmid contained in the bacterial cell that carries most of the functions for DNA transfer. There are two important components in the Ti plasmid. One is called T-DNA (transferred DNA) and has been shown to be transferred to and stably incorporated in the nuclear DNA of cells transformed during the infection process. Although the T-DNA is the mobile element, it does not itself encode the products that mediate its movements. It is a second component, the Ti plasmid virulence (vir) region, which provides most of the trans-acting products for T-DNA transit (DeBlock *et al.* 1984, Klee *et al.* 1987, Schell 1987, Gasser and Fraley 1989, Zambryski 1992).

The T-DNA is defined by flanking 25-base pair (bp) directly repeated sequences, which are responsible for the definition of the region that is to be transferred to the infected plant cell (Wang *et al.* 1984). Any DNA placed between the borders will be transferred to a plant. Transfer of T-DNA has been shown to be polar; deleting or reversing the orientation of the right T-DNA border abolishes T-DNA transfer, while manipulations of the left border have little effect. Expression of the genes located on the T-DNA leads to the formation of proteins involved in the production of indole acetic acid (an auxin) and isopentenyl-AMP (a cytokinin). These plant hormones cause the tumorous phenotype that is characterized by the ability of the plant cells to proliferate limitlessly and autonomously even in the absence of added phytohormones. These tumors are characterized by the production of opines. The biosynthesis of opines is catalyzed by opine synthases, which are encoded by the T-DNA. Based on the kind of opines produced in the tumors, agrobacteria are classified as octopine, nopaline, succinamopine, and leucinopine strains. The opines formed in the tumors can be metabolized by the tumorigenic agrobacteria, but not by most other soil organisms. Thus, *Agrobacterium* creates a favorable niche for itself by genetic modification of plant cells (Zambryski *et al.* 1989, Zambryski 1992, Hooykaas and Beijersbergen 1994).

Three important elements in *Agrobacterium* are essential for plant transformation by T-DNA: T-DNA border sequences (Wang *et al.* 1984), *vir* genes (Stachel *et al.* 1986, Stachel and Nester 1986, Citovsky *et al.* 1992) and chromosomal virulence genes *(chv)* (Douglas *et al.* 1985). An early step in the process of tumor induction is the attachment of agrobacteria to plant cells at the wound site. This step occurs concomitantly with or prior to vir gene- induction. The genes involved in attachment are located in the *Agrobacterium* chromosome, and include *chvA*, *chvB*, *pscA* (or exoC) and *att* (Douglas et al. 1985) .

The Ti plasmid vir region provides most of the products that mediate actual T-DNA movement. Plants susceptible to *Agrobacterium* infection produce low-molecular-weight phenolic compounds when wounded; such compounds are recognized by *Agrobacterium* as signal molecules that specifically induce expression of the vir genes. It is the expression of the vir genes that initiates the mobilization and transfer of the T-DNA (Stachel and Nester 1986, Stachel et al. 1986, Citovsky et al. 1992).

Vir genes have been reported to be induced by phenolic compounds such as acetosyringone (Stachel *et al.* 1985, Bolton *et al.* 1986, Yasumori *et al.* 1986, Sheikholeslam and Weeks 1987, Mathews *et al.* 1990), by incubation conditions such as low pH (Stachel *et al.* 1986, Sheikholeslam and Weeks 1987, Winans *et al.* 1988), by lignin precursors such as coniferyl-alcohol (Spencer and Towers

1988, Shimoda *et al.* 1990), by synergistic effects of phenolic compounds and sugars (Cangelosi *et al.* 1990, Shimoda *et al.* 1990), and also induced by starvation of inorganic phosphate (Winans *et al.* 1988).

The vir region is 30 kbp and organized into seven complementation groups, vir A, vir B, vir D, vir G, vir E, vir C, and vir H (Zambryski 1992). Some vir genes are essential for T-DNA transfer while others enhance the process. A first group of vir genes are required for the generation of a T-DNA copy. Vir A and vir G form part of a sensor/regulator component that responds to the presence of phenolic compounds and activates other vir regions. The products of vir D, vir D1 and vir D2 act as site-specific endonucleases that recognize and cleave the lower strand of the T-DNA border sequences; vir C enhances this activity. Products of a second group, vir E and vir D2, form the T-DNA strand-protein complex. These two gene products may be sufficient to protect and unfold the T-strand, and to provide SSB (single stranded DNA binding protein) functions. A third group, vir B products, has been suggested to be localized in the membrane and periplasm of *Agrobacterium*, providing a channel structure through the bacterial membrane. This localization would allow these proteins to play a role in T-strand complex export. There is a fourth group of vir products suggested to be required for targeting of the T-complex into and within the plant cell as well as for integration of the T-strand into plant cell DNA. These are vir D2 and vir E2 which provide nuclear localization functions that import, direct and assist in the integration process of the T-strand into the plant cell nucleus (Zambryski 1992, Hooykaas and Beijersbergen 1994).

Agrobacterium strains that induce tumor growth are known as wild type strains. In order to have a reliable plant transformation system, elimination of the phytohormone coding genes that induce aberrant cell proliferation is needed. The contents of T-DNA itself have no effect on the efficiency of T-DNA transfer.

Hence non-oncogenic derivatives of the *Ti* plasmids, where most of the internal sequences of the T-DNA have been replaced, are widely used as vectors for genetic transformation of plant cells.

A critical step in the development and evaluation of transformation strategies for plants is the availability of plasmid vectors with genes that can act as dominant selectable markers. Under various selection pressures, these genes provide a growth advantage to cells that integrate and express them. Without an efficient selection system, the transformants would be difficult to detect and could not be recovered or separated from the untransformed cells. A number of selectable marker genes have been developed that provide resistance to a range of antibiotics and chemicals; such compounds can arrest the growth of non-transformed cells or slowly kill them. The most widely used selectable marker is *NPTII* from the bacterial transposon Tn5 which codes for the enzyme neomycin phosphotransferase. This enzyme confers resistance to kanamycin (Herrera-Estrella *et al.* 1983). Other selectable markers are chloramphenicol acetyltransferase (*CAT*), dihydrofolate reductase (*DHFR*), hygromycin phosphotransferase (*HPT*) and *Streptomyces hygroscopicus* bar genes which encode resistance to chloramphenicol (DeBlock *et al.* 1984), methotrexate (Gritz and Davis 1983), hygromycin (Vander Elzen *et al.* 1985) and phosphinothricin (PPT, Somers *et al.* 1992), respectively. To achieve constitutive expression in culture and in the various tissues of transgenic plants, the coding regions of the genes for the markers have been fused to promoters and other regulatory sequences known to function in plants such as those from the nopaline synthase (*nos*) or octopine synthase (*ocs*) genes of the *Ti* plasmids.

Reporter genes are not used for selection, but code for enzymes that can be easily detected with great sensitivity. They are important for monitoring transformation or for detection of promoter activity. The opine synthase genes of the *Ti* plasmid, *nos* and *ocs*, and the chloramphenicol acetyltransferase (*cat*) gene from bacteria are good examples of reporter genes. Assays for the expression of these genes can be performed with cell-free extracts of transformed tissues and the opines can be detected by staining or fluorography after paper electrophoresis.

A relatively newly developed reporter gene is one coded for by the *E. coli* β-glucuronidase gene (*gus*), which allows great sensitivity of detection. ß-glucuronidase is a hydrolase that cleaves a variety of β-glucuronides including fluorogenic substrates that can be detected by fluorometry. β-glucuronidase (GUS) activity can be detected histochemically in cells using substrates such as X-gluc (5-bromo-4-chloro-3-indolylglucuronide). The GUS gene expression can be accurately measured on very small amounts of transformed plant tissues (Jefferson 1987).

Agrobacterium based vectors for plant transformation are basically of two types; cointegrating vectors that cointegrate into a resident *Ti* plasmid, and binary vectors that replicate autonomously. That is, in the cointegrating system, the *Agrobacterium* strain carries a disarmed and engineered *Ti* plasmid while in the binary system the *Agrobacterium* contains a *Ti* plasmid with disarmed or deleted T-DNA providing the *vir* functions and a separate vector providing the engineered T-DNA. In either situation there are some general components of the vector plasmid needed in the genetic engineering of plants such as (1) at least one border of the T-region, (2) dominant marker that is selectable for the transformed plant tissue, (3) sequences that permit replication of the plasmid in bacteria, (4) a marker for maintenance and selection in *Agrobacterium* and *E. coli* and, (5) foreign genes with promoters functional in plants (constitutive or inducible). These days binary systems are used almost exclusively because they are so much easier to manipulate (Gruber and Crosby 1993).

With both kinds of vector systems, the simplest way to obtain transgenic plants is to infect tissue explants with the manipulated *Agrobacterium* and to select for transformants in culture. Virtually all current transformation protocols with *Agrobacterium* vectors involve three basic steps: a) incubation of wounded plant tissue with the engineered *A. tumefaciens*/ vector combination; b) removal of the vector bacteria with a non-selective antibiotic *in vitro*; and c) regeneration of transformed plants *in vitro* in the presence of the selective agent. Regenerated shoots are in turn excised from the explant and transferred to a new medium for root induction. At all stages after plant-bacterium co-cultivation, a selection pressure is maintained on the regenerating plant tissues so that in theory only true transformants will be generated. In practice, however, selection systems are rarely 100% efficient, and some 'escapes' may emerge. For this reason, a detailed molecular analysis of putative transformants must be carried out to demonstrate the integration of foreign DNA sequences in the plant genome (Gruber and Crosby 1993).

Agrobacterium-mediated transformation has been achieved with a variety of cell types including protoplasts, callus tissue and cells from leaf disks. Generally, the recipient cell or tissue source employed depends largely on their capabilities for plant regeneration which varies greatly among species.

Particle Gun Transformation

The biolistic method is a direct gene transfer method. DNA delivery into cells by particle bombardment is a relatively recent development in transformation technology (Klein *et al.* 1987, Sanford *et al.* 1987). It is based on the acceleration of microprojectiles (0.4 to 4 µm) to speeds (300 to 600m/s) sufficient for the penetration of plant cell walls and membranes, which are the principal barriers to DNA delivery. Sanford (1990) defines the process as the introduction of substances into intact cells and tissues through the use of high-velocity microprojectiles. A microprojectile is any small coherent particle capable of being accelerated, such that it penetrates cells and tissues. It should be small enough to enter without detriment to the target, and should be capable of carrying DNA on its surface. Usually microprojectiles are made of high-density metals such as tungsten or gold, and are more or less spherical. Currently, biolistic devices primarily employ macroprojectiles as a means for accelerating microprojectiles. There are several factors that influence the efficiency of DNA delivery into target tissue (Klein *et al.* 1988a) such as size of particle, target size, DNA quantity, microprojectile volume, number and size of microprojectiles, vacuum in the chamber, distance to the plant cell or tissue, and presence of $CaCl_2$ and spermidine.

There have been a number of publications where successful plant transformation was accomplished via microprojectile bombardment such as in soybean (McCabe *et al.* 1988), tobacco (Klein *et al.* 1988b), papaya (Fitch *et al.* 1990, 1992) and hybrid poplar (McCown *et al.* 1991). Monocot species that are recalcitrant to *Agrobacterium*-mediated DNA transfer have been transformed with this method such as wheat (Vasil *et al.* 1992), corn (Gordon-Kamm *et al.*1990) and oat (Sumers *et al.* 1992).

Cross Protection

Plant viruses cause significant losses to most, if not all, major crops around the world. There are several ways by which virus diseases are being controlled (Hull and Davies, 1992). One, which is applied primarily to perennial crops, is the use of healthy planting material and/or the eradication of infected plants. The main disadvantages of this approach are difficulties in the detection of infected plants, and the economic and social difficulties of enforcing eradication schemes. Another method, which is applied to annual crops, involves various cultural practices. This approach can fail if there are changes that affect virus epidemiology such as local climatic fluctuations, or changes in cropping practice. A third approach is breeding resistance to the virus or its vector into the crop and it is regarded as being the best in the long term. However, it has some drawbacks, such as that genes for resistance may not exist or resistance may be polygenic and therefore difficult to fix by crosses.

Although it is difficult to delineate the specific levels of resistance displayed by plants to virus infection into discrete categories, there are some terms which are commonly used to describe host-plant reactions to virus infection. If, upon entry of the virus into a host plant, the virus fails to replicate in the initially infected cells, the plant is considered 'immune' to the virus. However, if the virus is able to multiply in the initially infected cells but fails to move to adjacent cells, the plant is considered to be 'resistant'. The resistance can be due to reduction in the replication of the virus, its inability to move to adjacent cells and other parts of the plant, or both. A plant is considered to be 'susceptible' if the virus is able to replicate in the initially infected cells, spread to adjacent cells and then to other parts of the plant and replicate in uninoculated leaves (Pappu *et al.* 1995).

A brief description of the life cycle of a typical positive-sense, single-stranded RNA virus is given by Pappu *et al.* (1995). Plant viruses are passively introduced into plant cells by mechanical means or by vectors. Upon entry, the virion is uncoated, releasing the viral RNA. The viral RNA is then translated in the 5' to 3' direction, initially to synthesize the gene products required for the replication of the RNA by the RNA-dependent RNA polymerase and possibly utilizing both viral and host-encoded components. Plant viruses employ different strategies, singly or in combination, for the expression of the various viral genes: readthrough; translational frameshift; polyprotein processing; production of subgenomic RNA; and divided genomes. These strategies are discussed in detail by Matthews (1991). In addition to the structural genes, such as that encoding the coat protein required for packaging of the progeny viral RNA, additional non-structural genes, coding for movement protein, protease, and helper component/acquisition factor for vector-transmission, are synthesized at various stages in the viral life cycle. In the final phase, progeny viral RNA are packaged into virions by the coat-protein subunits.

Hull and Davies (1992) consider cross protection another form of resistance, or induced resistance. Cross protection is "the use of a mild virus isolate to protect plants against economic damage caused by infection with a severe challenge strain(s) of the same virus" as defined by Gonsalves and Garnsey (1989). Cross protection has been used successfully in various crops, e.g., in papaya against papaya ringspot virus (PRSV) (Yeh *et al.* 1988) and in citrus against citrus tristeza virus (CTV) (Muller and Costa 1977, Costa and Muller 1980, Gonsalves and Garnsey 1989).

While the various types of resistance generally provide the best prospects for virus control, there are several problems that have to be considered. In tolerant varieties and in cross protection, the virus multiplies in the host and thus can provide sources of inoculum for other crops in which the reaction may be more severe. Furthermore, virus multiplication can lead to virus variation, which could give rise to more virulent isolates.

Coat Protein-Mediated Protection

The presence of one virus in a plant can interfere with infection by another virus or strain, resulting in cross protection. The idea that individual viral components contained in plants might also interfere with virus infection was developed in 1985 by Sanford and Johnston. They stated that certain key gene products of a pathogen present in the plant in a dysfunctional form, in excess, or at the wrong time of viral development could disrupt infection by the invading pathogen. They termed this concept as parasite-derived resistance. This term is most recently referred to as pathogen-derived resistance (Lindbo and Dougherty 1992a).

Recent scientific advances led to the development of the concept and its application to conferring protection against plant viruses. First, there were major advances in the methods of introducing foreign nucleic acids into plant cells and in the regeneration of transformed plants from these cells. Transformation systems were developed for a range of dicotyledonous species and for some important monocotyledons including certain lines of rice, maize, barley, and wheat. Also, there was a great expansion in the understanding of the genome organizations and gene functions of many of the groups of plant viruses.

In the last decade, transgenic plants carrying nucleotide sequences derived from plant viruses have shown different levels of resistance to infection or suppression of disease symptoms. Transferred genes include segments of viral genomes encoding capsid or coat proteins, sequences encoding proteins that are or may be subunits of the viral replicase, sequences incapable of encoding proteins, entire genomes of defective interfering viruses and satellite viruses, and complete genomes of mild strains of virus (Fitchen and Beachy 1993).

Pathogen-derived resistance was first successfully achieved in transgenic tobacco plants protected against tobacco mosaic virus (TMV). The transformed plants had a chimeric gene containing a cDNA copy of the coat protein (CP) gene of TMV (Powell-Abel *et al.* 1986). These plants were found to have an increased level of resistance to TMV infection; the transgenic plants that expressed the CP escaped infection while those plants that lacked the CP gene developed disease symptoms. It was proposed that, even though the virus particle shape and the replication strategy are different with each virus, expression of the capsid protein gene in transgenic plants provides resistance against the virus from which the gene was isolated. Thus, this represents a generic method to produce virus resistance. The phenomenon was given the name coat protein-mediated resistance.

The CP is a high molecular weight molecule, organized in subunits called capsomeres which surround the nucleic acids of the virus. The CP not only protects the viral genome, but is also the site of interaction with the host organism at several stages of the viral cycle. CP functions are quite variable depending on the virus, but can play a direct role in replication of the viral genome (alfalfa mosaic virus), movement of the virus within the plant (e.g., tobacco mosaic tobamovirus (TMV), and recognition by host resistance functions (TMV on *Nicotiana* species of N' genotype). The CP also plays an essential role in virus transmission, in that it is the site of recognition necessary for transmission by vectors such as insects (Matthews 1992, Tepfer 1993).

Different instances of coat protein-mediated resistance have been reported. Powell-Abel *et al.* (1986) reported that the level of resistance to infection was directly related to the level of expression of the gene and the accumulation of CP. It was suggested that the CP of the protecting virus inhibited the uncoating of the incoming strain of that virus. The proportion of plants that remained without symptoms and the duration of the delay in symptom development in those that did become infected were reduced when the concentration of virus in the challenge inoculum was increased. Moreover, when TMV RNA rather than TMV was included in the inoculum, infections were initiated at roughly the same frequency on CP protected plants (CP^+) and CP nonprotected plants (CP^-), and similar proportions of CP^+ and CP^- plants developed systemic disease (Nelson *et al.* 1987).

There is a rough correlation between the degree of similarity between the CP of the challenge virus and TMV and the extent of resistance conferred by the TMV-CP transgene, as shown by Nejidat and Beachy (1990) and Anderson *et al.* (1989). TMV-CP transgenic plants were tested for resistance to infection by another strain of TMV, by other viruses in the tobamovirus group, and by viruses belonging to other groups. Protection against a closely related strain was essentially as effective as homologous protection. Protection against other tobamoviruses depended upon which tobamovirus was included in the inoculum and its apparent degree of relatedness to TMV according to CP sequence. The CP of tomato mosaic virus (ToMV) shares 82% amino acid identity with the TMV CP, and tobacco mild green mosaic virus (TMGMV) shares 73% identity. These viruses were similar to TMV in the extent to which the plants were able to resist infection. In contrast, ribgrass mosaic virus (RMV), the CP of which is only 45% identical to TMV CP, was much more able to infect and cause disease in CP+ plants. Protection against viruses belonging to other groups was very weak or nonexistent.

Sanford and Johnston (1985) suggested that distantly related parasites do not cross protect because their proteins might have different binding sites during their replication cycles. They suggested that something (perhaps some product) in the first protecting parasite blocks replication of the second parasite; for example, the replicase of the first virus might bind to the parasite replicase binding site, inhibiting or blocking replication of the second parasite.

In contrast to the protection conferred against TMV by TMV-CP, transgenic potato plants accumulating very low levels of CP of potato virus Y (PVY) strains PVY^0 and PVY^N exhibited a high degree of resistance to infection when challenged by mechanical inoculation (Kaniewski *et al.* 1990). To further demonstrate a lack of a positive correlation between coat protein levels and protection, a gene lacking an initiating methionine codon was constructed. Transgenic plants carrying this gene were as resistant to PVY infection as were plants carrying the construct with the initiation signal present. In another instance, transgenic plants with the CP of tobacco etch virus (TEV) were obtained (Lindbo and Dougherty 1992a, 1992b). Gene constructs to direct the synthesis of full-length as well as truncated forms of the CP were made, as were constructs to produce an antisense transcript and a transcript without the added translational initiation site. The transgenic plants were challenged by mechanical inoculation with relatively high levels of purified virus or virus-containing plant sap. Transgenic plant lines expressing truncated genes appeared to have more effective resistance than lines expressing the entire gene, although only a few lines were analyzed and the variation between lines was great. Much more striking was the delay in the appearance of symptoms, and the amelioration of symptom severity, in plant lines accumulating only CP-related RNA sequences. Many individual plants of both an antisense plant line and a plant line expressing the gene construct without the initiating methionine codon remained entirely asymptomatic and did not accumulate virus. The resistance was independent of inoculum concentration over the range used. However, the resistance was highly specific; resistance was not conferred against infection by other potyviruses.

Resistance to TMV in transgenic TMV CP$^+$ tobacco is the most thoroughly studied system. In this case, several mechanisms are believed to contribute to the resistance. A major component of coat protein-mediated resistance must result from an action of the transgene-derived coat protein on an early event in the infection process because the number of infectible sites in the inoculated leaf, as assessed by lesion production following mechanical inoculation, is lower on CP$^+$ plants than on control plants (Nelson *et al.* 1987). This impaired step probably occurs after entry of the virus and prior to completion of the normal process of uncoating (removal of the protein coat). It has been suggested that the introduced CP may interfere with or prevent the uncoating process of invading viruses, a process which is an essential precursor to nucleic acid replication and protein synthesis by plant viruses. It has also been reported that the level of resistance to infection was directly related to the level of expression of the gene and the accumulation of CP (Powell-Abel *et al.* 1986). However, this is not always the case. There are an increasing number of examples where there is no apparent relationship between resistance and the steady-state accumulation of transgene transcript or encoded protein product. While the production of CP is essential for protection against TMV, just the mRNA of the CP was found sufficient to confer protection against other viruses (Fitchen and Beachy 1993, Lindbo *et al.* 1993). When the degree of protection does not correlate with levels of gene expression, the low frequency may indicate that some other, unidentified criterion plays an essential role in resistance. The transgene may act on initiation of infection, replication of virus, spread of the infection throughout the plant, or symptom development. Another group working with transgenic tobacco plants with TEV genome ligated to a mutated version of the TEV CP gene sequence that rendered it untranslatable obtained different responses in transgenic plants when inoculated with TEV: highly resistant plants, susceptible plants that were able to recover from systemic TEV infection and susceptible plants (Dougherty *et al.* 1994). They suggested that the resistance observed in the highly resistant plants is more likely mediated by a cellular pathway involved in the targeted elimination of unspliced or aberrant mRNAs instead of subscribing to the hypothesis that the trangene product interacts directly with the virus. Transgenic plants displaying the recovery phenotype likely manifest the resistance using a similar mechanism.

It is clear that the virus resistance phenotypes reported are often quite varied, and the underlying mechanisms of resistance are not well understood. Recently it has been reported that it is highly unlikely that a single mechanism accounts for resistance in all cases. Indeed, the resistance derived from a single CP gene in a single plant species may act by more than one mechanism and may inhibit several different stages in the infection or replication process (Fitchen and Beachy 1993).

The main advantages of engineered cross protection over classical cross protection are that protected plants do not contain infectious virions, the protective effect (coat-protein expressing trait) is transferable to progenies, and the possibility of mild strains mutating to severe strains is reduced in engineered cross protection. One major, but still controversial, disadvantage of CP-mediated protection is that it could change the epidemiology of heterologous viruses and even more there is the question of the potential impact of recombination between transcripts of viral genes and infecting viruses (Tepfer 1993). It is possible that new viral strains (and diseases) could be created by heterologous encapsidation or by the recombination of incoming viral RNA with transgenic mRNA. Heterologous encapsidation takes place naturally when two virus strains infect the same plant and the genomic RNA of one strain becomes totally or partially encapsidated by CPs of the other strain. This phenomenon is defined as transcapsidation in the first case, and a mixed encapsidation or phenotypic mixing in the second case. In potyviruses, transcapsidated particles have shown altered ways of transmission. Bourdin and Lecoq (1991) report that the non-aphid-transmissible isolate of zucchini yellow mosaic virus (ZYMV), which is transmission-deficient on the CP, is transmitted when in a mixed infection with an aphid-transmissible isolate of PRSV and they provide evidence that this phenomenon occurs through heteroencapsidation between the two potyviruses. Heterologous encapsidation was also reported to occur in potato plants transgenic for the coat protein gene of potato virus Y strain N (PVY^N) when infected with PVY^O (Farinelli *et al.* 1992).

Another type of risk when using on *A. tumefaciens* transformation system has been reported (Mogilner *et al.* 1993). These reseachers followed the survival of the engineered bacteria, carrying a mini-Ti plasmid with a dimeric cDNA of citrus exocortis viroid (CEVd), in the inoculated hosts. They found that, 38-90 days after inoculation, agroinfected plants still contained large amounts of the engineered bacteria and suggested the need for more stringent quarantine measures when handling *A. tumefaciens* cells harboring constructs for agroinoculation with plant viruses or viroids.

Citrus Tristeza Virus

Citrus tristeza virus (CTV) is distributed worldwide and causes economically important diseases wherever citrus is grown (Bar-Joseph *et al.* 1989, Bar-Joseph and Lee 1990). The virus can cause a variety of effects, including stunting, slow decline, quick decline, stem pitting, or no symptoms, depending on the virus isolate, environmental conditions, citrus scion cultivar, and rootstock. However, the most destructive damage is the induced decline in scions grafted on sour orange (*Citrus aurantium* L.) rootstock and the severe stem pitting and loss of plant vigor on some oranges and grapefruit regardless of the rootstock (Garnsey 1987, Garnsey and Lee 1988).

CTV, a member of the closterovirus group, has the largest positive ssRNA genome, estimated to be 20 Kb, among plant viruses studied to date (Bar-Joseph and Lee 1990). Virions consist of long flexuous filamentous particles, encapsidated by a single type of CP of 25-kDa (Lee *et al.* 1988). The virus is phloem-limited and aphid transmitted, in a semi-persistent manner. CTV can be transmitted by grafting and mechanically by slash inoculating partially purified virus into the stem of receptor citrus host. Seed transmission has not been demonstrated (Bar-Joseph *et al.* 1989, Bar-Joseph and Lee 1990). Cloning and sequencing of the CP gene of CTV revealed a 669 nucleotide open reading frame, and confirmed two degradation products (CP1 and CP2) that arise from the primary CP gene product, probably by post-translational proteolysis (Sekiya *et al.* 1991). To date, the CTV genome has found to be complex as well as large. Pappu *et al.* (1993) sequenced the 3' 7292 nucleotides and described eight open reading frames: ORF1, p65; ORF2, p61; ORF3, p27; ORF4, p25; ORF5, p28; ORF6, p13; ORF7, p20; and ORF8, p23. The 65-kDa protein is a viral homolog of cellular hsp70 heat shock proteins (hsp), and the 61-kDa protein is distantly related to the hsp90 proteins. The 27-kDa protein is a diverged copy of the CP and is located immediately upstream of the CP gene. The 25-kDa protein corresponds to the CP. Four ORFs were found downstream of the CP: p18, p13, p20 and p23. The possible roles of these four genes in infection are not known. More recently, further extension of the CTV genome revealed four additional ORFs (Dolja *et al.* 1994): putative methyl-transferase (MTR), and helicase (HEL) domains, the putative RNA-dependent RNA polymerase (POL) domain, and a 33-kDa protein of unknown function.

CTV exhibits the phenomenon of cross-protection as demonstrated in Brazil (Costa and Muller 1980), where infection with mild isolates as a strategy to reduce losses due to CTV has been commercially used to control CTV-stem pitting isolates on sweet oranges. The same strategy is a part of South Africa's citrus cultivar improvement program to reduce stem pitting injury in grapefruit (Bar-Joseph *et al.* 1989).

In order to use the genetically engineered cross-protection system in CTV, it was first necessary to mobilize the coat protein gene to a plasmid vector that could be used for plant transformation. These needs were achieved in Dr. K. Cline's lab, University of Florida. A transformation vector containing the CTV coat protein (CTV-CP) was constructed utilizing the pUC18CPexp and pGA482GG system described by Slightom *et al.* (1991) and the T-36 CTV-CP gene isolated and cloned by Sekiya *et al.* (1991).

Citrus Transformation

For many fruit and nut crops, cultivars carrying novel traits cannot be developed within the span of a breeder's career. Juvenility periods range from one year for raspberry to 15 years for pecan (Sherman and Lyrene 1983). Peach, a crop with a relatively short generation time of 3 to 4 years, has generally required 20 years from first fruiting to cultivar release. Using genetic transformation techniques in conjunction with conventional breeding in fruit trees which usually are highly heterozygous, have an extended sexual generation and are propagated mainly vegetatively, represents a major advantage since a specific trait may be added to a given cultivar or rootstock genome while avoiding burdens of sexual recombination. Thus perennial fruits and nuts may represent the crops that, in the long term, could harvest the greatest benefits from gene transfer technologies (Scorza 1991).

Plant cell and tissue culture techniques provide a basis for heritable modification of plants since somatic cells or tissues of many species can be manipulated in culture, then induced to regenerate mature, fertile plants from a single altered cell. Research on *Citrus* has shown that it is responsive in tissue culture and several *in vitro* regeneration systems have been developed (Murashigue and Tucker 1969, Spiegel-Roy and Vardi 1984, Litz *et al.* 1985, Beloualy 1991). Several *Citrus* transformation protocols have been reported, based on the introduction of naked DNA into protoplasts derived from embryogenic calli (Kobayashi and Uchimiya 1989, Vardi *et al.* 1990, Hidaka and Omura 1993) and on *Agrobacterium tumefaciens*-mediated transformation of cells (Hidaka *et al.* 1990) and stem segment explants (Moore *et al.* 1992, Kaneyoshi *et al.* 1994, Peña *et al.* 1995a, 1995b). All methods give low levels of transgenic plant production compared to species such as tobacco.

All necessary components, were in place to study improvement of transformation efficiencies and CP-mediated protection against CTV in citrus.

CHAPTER 3
GENETIC TRANSFORMATION OF CITRUS PLANTS

Introduction

As discussed above, it has been shown previously that it is possible to produce transgenic *Citrus*, although the procedure has not been optimized. Genetic transformation of plant genomes with viral coat protein genes has been shown to confer a type of cross protection (CPMP) to the previously susceptible plants. In addition, a cDNA to the citrus tristeza virus (CTV) coat protein gene (CTV-CP) was generated that can be used to obtain transformed citrus plants (Sekiya 1990). This CTV-CP cDNA was available to initiate such experiments. This cDNA may confer the novel type of resistance seen in tobacco plants and other crops to suitable citrus cultivars.

The objectives of this study were to evaluate different factors that affect the frequency of *Citrus* transformation and to obtain transformed plants containing the citrus tristeza virus coat protein gene that could be used to evaluate the cross protecting ability of this transgene in plants.

Materials and Methods

Plant Material

Carrizo citrange [*C. sinensis* (L.) Osb. x *Poncirus trifoliata* (L.) Raf.], Key lime [*C. aurantifolia* (Christm.) Swing.], Brasilian sour orange [*C. aurantium* L.] and Ridge Pineapple sweet orange [*Citrus sinensis* (L.) Osb.] were used in the transformation experiments. Citrus seeds were purchased from Willits & Newcomb (PO Box 428, Arvin CA 93203). Seeds were disinfected for 10 min in 70% ethanol, then for 20 min in 20% Clorox + 2 drops of Tween-20 per 100 ml, and were then rinsed one minute 3 times with sterile distilled water (Moore *et al.* 1992). Seeds were placed individually in 150 x 25mm tubes containing half-strength MT medium (Murashige and Tucker 1969) where they germinated aseptically. The cultures were maintained at 27°C with 16 hr of cool-white fluorescent light (76 μmol s^{-1} m^{-2}).

Vector Plasmids

Two vector plasmids were evaluated-pMON9793 and pGA482GG. The binary transformation vector pMON9793 was generously provided by the Monsanto corporation. Features of this plasmid include a single (right) T-DNA border and chimeric genes for the selectable marker neomycin phosphotransferase-II (NPTII) and the scorable marker β-glucuronidase (GUS). The NPT-II gene has nopaline synthase (NOS) promoter and termination sequences; the GUS gene has been provided with a mannopine synthase (MAS) promoter and NOS 3′ sequences (Figure 3.1).

Plasmid pGA482GG, kindly provided to us by J. Slightom of UpJohn, contains both left and right T-DNA border fragments (BL and BR); a restriction enzyme polylinker, a NOS/NPTII/NOS gene, a cauliflower mosaic virus 35S (CaMV35S)/GUS/NOS gene within the T-DNA region, and gentamycin and tetracycline genes for bacterial selection outside the T-DNA region (Figure 3.1).

A transformation vector for the introduction of CTV coat protein (CP) gene into plants was constructed by Mike McCaffery in Dr. Ken Cline's laboratory, in Horticultural Sciences Department at the University of Florida, utilizing the pUC18CPexp and pGA482GG system described by Slightom (1991). The 669bp reading frame of the T-36 CP gene (Sekiya *et al.* 1991) was PCR cloned and placed into the *NcoI* site of a cloning cassette in pUC18CPexp. The cassette containing the CP construct was then cloned into the *HindIII* site in the polylinker (An 1986, Fitch *et al.* 1990), to produce pGA482GG-CP.

Agrobacterium Strains

Disarmed *A. tumefaciens* strain EHA101 and wild type strain A518 were evaluated. The *A. tumefaciens* strain EHA101 (Hood *et al.* 1986) was used in many experiments. This strain carries the hypervirulent, tumor-inducing plasmid pTiBo542 from which T-DNA sequences have been deleted. A bacterial kanamycin resistance gene for bacterial selection is also present on this plasmid. A518 is an *Agrobacterium* strain with a wild-type T-DNA plasmid (Sciaky *et al.* 1978) that causes shooty tumors in *Citrus*. These bacterial strains were kindly provided by E. Hood and B. Gurley, respectively.

The binary vector plasmids pMON9793 and pGA482GG-CP were introduced individually into EHA101 by direct DNA uptake (An 1987). pGA482GG-CP was also introduced into A518 .

Transformation

Nodes and internodal stem segments were cut from 3-4 month old seedlings grown *in vitro*. Explants were placed on regeneration medium [full strength MT medium, sucrose 5%, 6-benzyladenine (BA) 0.5 - 5.0 mg/L, 0.8% agar and pH 5.7]. Between 20 to 30 explants were evenly distributed in 100 X 15mm disposable petri plates (Falcon, Lincoln Park, N.J.). The stem segments were oriented with either the basal

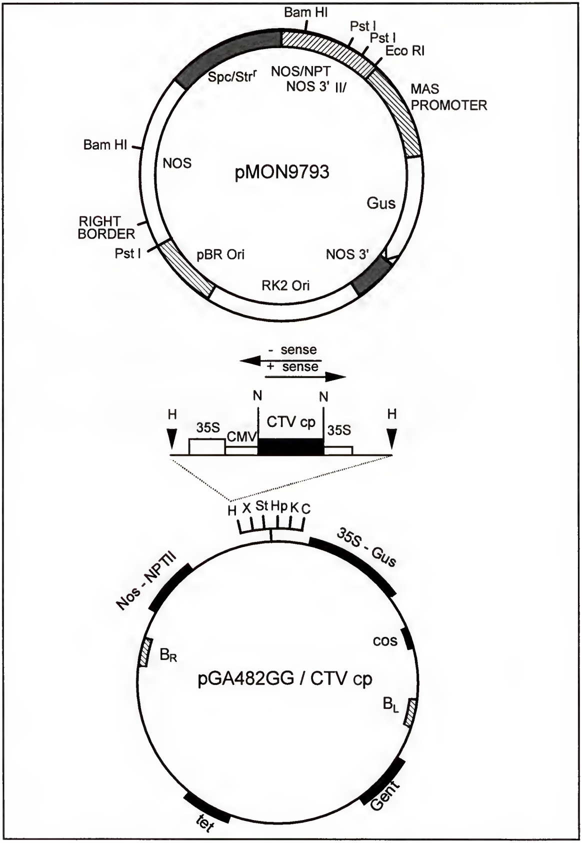

Figure 3.1. Vector plasmids evaluated for citrus transformation experiments.

(B↑) or apical (A↑) end of the segment protruding from the medium or horizontally (hor).

Cultures of both *Agrobacterium* strains to be used for transformation experiments were initiated from glycerol stocks and grown overnight at 28°C in YEP medium (An 1987) containing the appropriate antibiotics as described by Moore *et al.* 1992. Target organs were inoculated with overnight *Agrobacterium* cultures by placing a syringe drop of bacterial culture on top of the explant.

Transformation via Particle Bombardment Followed by *Agrobacterium* Inoculation

This approach was tested to determine whether wounding via particle bombardment prior to inoculation would enhance *Agrobacterium* transformation frequencies (Bidney *et al.* 1992). The method consisted of creating wounds by the impact of particles on either stem segments or node segments of key lime in the hope of producing sites conducive to *Agrobacterium* infection over much of the surface area, followed by inoculation. The biolistic device used was Model PDS-1000/He Biolistic™ Particle delivery system BIORAD (™ of E.I. DuPont de Nemours & Co.).

The conditions for the bombardment were as follows: 400 psi, 650 psi, 900 psi, 1100 psi, 1500 psi and 1800 psi size rupture disks were tested with 1.11 μm tungsten particles (60mg/ml) and 28in Mg vacuum. Stems or nodes, around 20 at a time, were placed to fill a 1-cm^2 area in the middle of a disposable petri plate containing regeneration medium. The plate was positioned in the third level of the chamber and shot twice with 10 μl particle suspension.

Samples were then immediately inoculated with overnight grown *Agrobacterium* vector EHA101-pGA482GG-CP by placing a syringe drop of bacterial culture on top of the explant.

Selection and Regeneration

Explants were co-cultivated with *Agrobacterium* for 1-3 days then transferred to regeneration medium supplemented with 200 μg/ml mefoxin (mefoxin= cefoxitin sodium, Merck, Sharpe & Dohme) to inhibit further growth of bacteria and 100 μg/ml kanamycin for selection. In a few experiments, two weeks after inoculation, a thin overlay (7ml) of 100 μg/ml kanamycin in water was added to inhibit further growth of nontransformed cells (Serres *et al.* 1992). Four weeks later, shoots from nodes and stem segments were harvested for testing and the explants were transferred to fresh selection medium. Two more harvests of shoots were done at four week intervals, before discarding the nodes and stem segments themselves. Putatively transformed shoots were rooted in culture cups containing 50 ml of sterile potting soil moistened with 25 ml of half-strength MT.

Evaluation of Transgenic Shoots

All shoots that regenerated on selection medium were collected. A number of tests were conducted to distinguish transgenic shoots from those that had "escaped" selection.

ß-glucuronidase (GUS) assays. Basal portions of shoots regenerated at 4,8 and 12 weeks on selection medium were harvested and assayed histochemically for GUS activity (Jefferson 1987) following the protocol described by Moore *et al.* (1992). The surface of the section that stained blue was recorded either as sectored or entirely stained. Estimates of transformation frequencies were based on numbers and percentages of GUS positive (GUS$^+$) shoots produced.

Polymerase chain reaction (PCR). Subsequently PCR technique was used to detect specific T-DNA sequences from small amounts of putatively transformed plant tissue. Reaction components and concentrations were as previously described

(Moore *et al.* 1992). Samples were heated to 94°C for 4 min and then subjected to 30 cycles of 2 min at 94°C, 0.5 min at 55°C and 2.25 min at 72°C. Primers 5'GGT TTG AAC CAT GGA CGA CGA AAC AAA GAA ATT G-3' and 5'GGA ACT CCA CCA TGG CGA TAG AAA CCG GGA ATC GG-3' were used to PCR-amplify a 720 bp fragment of the CTV-CP. Primers to amplify 350 bp and 450 bp fragments corresponding to the NPTII gene (5'TCA CTG AAG CGG GAA GGG ACT-3' and 5'CAT CGC CAT GGG TCA CGA CGA-3'), and GUS gene (5' TTG GGC AGG CCA GCG TAT CGT-3' and 5'ATC ACG CAG TTC AAC GCT GAC-3') were also used.

Protein blot analysis. For immunoblot analysis, two antibodies to the CTV-CP gene were used: polyclonal antiserum No. 894 (kindly provided by Dr. D. Purcifull) prepared to sodium dodecyl sulfate degraded CTV strain T-4 coat protein (Garnsey *et al.* 1979), and monoclonal antibody MCA13 (provided by Dr. Garnsey) produced to strainT-36 (Permar *et al.* 1990). CTV is phloem-limited; therefore tissue samples consisting of bark, petiole or midribs from putatively transformed plants were collected and ground in a 1.5 ml eppendorf tube with a minipestle in the presence of liquid nitrogen. SDS-PAGE sample buffer (10% mercaptoethanol, 20% glycerol, 50mM Tris, 0.5% SDS) was added to the sample at a ratio of 1:4 (w/v). Protein blotting was performed as previously described by Sekiya (1990). During the immunostaining procedure, commercial goat anti-rabbit and goat anti-mouse IgG's (Boehringer Mannheim Biochemicals) labeled with alkaline phosphatase were used for antibodies 894 and MCA13 respectively, at the recommended dilution.

DNA blot analysis. DNA was extracted from approximately one gram of fresh bark and leaf tissue using a modified Dellaporta *et al.* (1983) procedure (Durham 1990). Restriction digests of genomic DNA and Southern hybridization were according to Maniatis *et. al* (1982). The probe was a 669bp *NcoI* → *NcoI* fragment containing the CTV-CP gene isolated from plasmid pGA482GG-CP and labeled with 32^P using a random primer labeling kit (GIBCO BRL). Genomic DNA from transformed plants was restricted with KpnI or *HpaI* (BRL enzymes) to generate an internal fragment and a border T-DNA fragment respectively. DNA analysis was conducted on Hybond-N nylon membranes (Amersham) and filters were washed under high stringency conditions (equivalent to 0.25X SSPE, 0.2% SDS at 65°C)

Results and Discussion

Citrus Transformation via *A. tumefaciens*

Transformation efficiency and production of transgenic plants was evaluated in four citrus cultivars. We evaluated different factors such as strain/plasmid combination, orientation of stem segments, concentration of cytokinin, use of wounding via particle bombardment and use of a culture overlay containing kanamycin.

Carrizo. Transformation experiments were done with Carrizo citrange, even though it is resistant to CTV, because it is a relatively easy citrus type to transform and previous data were available for comparison (Moore *et al.* 1992). Two types of explants were tested: internodal stem segments and node segments. Internodal stem segments were more readily transformed than node segments in all four cultivars evaluated (Tables 3.1 and 3.2). The percentage of GUS^+ Carrizo shoots produced from nodes varied between 2 and 8 in two experiments whereas the percentage for stem segments ranged from 2.9 to 33. Thus transformation of nodal segments was achieved, however with low frequencies. (Table 3.2). A transformation system of axillary buds in nodes may be very important because the potential for somaclonal variation seen in plantlets produced from adventitious buds (Larkin and Scowcroft 1981). Furthermore, it would be a great advantage to be able to transform non-juvenile tissue. According to Navarro *et al.* (1975), the plants that are derived from shoot tips of mature trees are also mature in phase; therefore shoots derived from axillary buds formed in mature nodes should also be mature. It seems reasonable to have obtained low frequencies of transformation when using nodal segments. *Agrobacterium* typically invades wounded plant cells in order to insert foreign genes into the plant genome, thus preformed meristematic cells represent a poor target for this method of transformation. Likewise, the higher levels of transformation seen in stem segments may be related to the organogenic events taking place while new adventitious shoots are being formed.

The greatest improvement in transformation frequency was due to the use of a particular vector plasmid. In first experiments using pMON9793, most shoots that regenerated on selective medium were "escapes", i.e. not transformed. This plasmid produced an average of 2.9% of GUS$^+$ shoots (Table 3.1). In later experiments, a much higher frequency of GUS$^+$ shoots was obtained with vector plasmid pGA482GG (33%). This difference can be attributed to the features of the plasmids themselves. For example, pMON9793 posseses a single (right) T-DNA border, while plasmid pGA482GG contains both left and right T-DNA border fragments, BL and BR (Fig 3.1). In these experiments, two *Agrobacterium* strains were evaluated, A518 and EHA101. Strain A518 was not as effective (12%) as strain EHA101 (33%) for production of GUS$^+$ shoots (Table 3.1).

Concentration of BA in the medium was also tested (0, 1 and 5mg/L). Fewer shoots regenerated in absence of BA, as expected, e.g. 1.2 compared to 1.9 shoots regenerated in 5mg/L. As a consequence, the total number of GUS$^+$

shoots produced in the experiment also decreased (from 8% to 3%). However shoots produced on low concentrations of BA were larger in size and rooted more easily than the smaller shoots produced on 5mg/L BA. From eleven GUS$^+$ shoots obtained in a single experiment using 1mg/L BA and stem segments (Table 3.1), eight shoots rooted and successfully adapted to soil whereas from 74 GUS$^+$ shoots produced using 5mg/L only two rooted and survived (data not shown). Finally, placing the stem segments horizontally on the medium greatly decreased the number of escape shoots produced, thus improving transformation efficiency.

Key lime. As with Carrizo, more GUS$^+$ shoots were generated when explants were inoculated with pGA482GG than with pMON9793 (Tables 3.1 and 3.2). Once it was clear that the pGA482GG vector plasmid was working and the CP gene was being expressed in transgenic Carrizo plants obtained previously, extensive transformation experiments were initiated with Key lime. Key lime transformation is of interest because this variety is an excellent indicator plant for the presence of CTV.

From the *Agrobacterium* strains evaluated, strain EHA101 was more effective for production of GUS$^+$ shoots when node segments were the explants, eg. between 0.6 - 7.0% shoots with EHA101 and only 0.3 - 3.3% with A518 (Table 3.2). Conversely, the use of strain A518 increased the number of GUS$^+$ shoots produced when stem segments were used as explants, e.g. from 0.2 - 6.8% with EHA101 to 6.9 - 22% with A518 (Table 3.1). Nevertheless, A518 is a wild type strain and integration of its wild type T-DNA inhibited rooting of transgenic shoots, which is already a major difficulty in producing transgenic citrus plants. Micrografting of the transformed shoots onto rootstocks was attempted to circumvent the problem, but the grafted shoots developed tumors in

TABLE 3.1. *Agrobacterium tumefaciens*-mediated transformation using stem segments from four citrus cultivars.

Agrobacterium strain and vector plasmid	Treatment [a]	No. stem segments cultured	No.(%) segments producing green shoots	Total no. shoots evaluated (avg. per seg. prod. shoots)	Avg. of shoots evaluated per seg. cultured	No.(%) GUS+[b] shoots
Carrizo citrange						
EHA101 (pMON9793)[c]	B↑, 5mg/L BA	1810	655(36)	1542(2.4)	0.85	44 (2.9)
EHA101 (pGA482GG)	B↑, 5mg/L BA	428	36 (8)	46(1.3)	0.11	15(32.6)
A518 (pGA482GG)	B↑, 5mg/L BA	417	14 (3)	16(1.1)	0.04	2(12.5)
EHA101 (pGA482GG-CP)[d]	B↑, 0mg/L BA	685	292(43)	362(1.2)	0.53	11 (3.0)
EHA101 (pGA482GG-CP)	B↑, 1mg/L BA	131	104(79)	253(2.4)	1.93	11 (4.4)
EHA101 (pGA482GG-CP)[e]	B↑, 5mg/L BA	1198	495(41)	927(1.9)	0.77	74 (8.0)
EHA101 (pGA482GG-CP)	hor,0mg/L BA	758	18 (2)	20 (1.1)	0.03	1 (5.0)
EHA101 (pGA482GG-CP)	hor,5mg/L BA	814	32 (4)	44(1.4)	0.05	11(25.0)
Key lime						
EHA101 (pMON9793)[e]	A↑, 2mg/L BA	1554	218(14)	551(2.5)	0.35	3 (0.5)
EHA101 (pGA482GG-CP)[e]	A↑, 0mg/L BA	1177	75 (6)	88 (1.2)	0.07	6 (6.8)
EHA101 (pGA482GG-CP)[e]	A↑,.5mg/L BA	1269	139(11)	345(2.5)	0.27	7 (2.0)
EHA101 (pGA482GG-CP)	A↑, 2mg/L BA	451	66(15)	218(3.3)	0.48	3 (1.4)
EHA101 (pGA482GG-CP)[f]	A↑, 2mg/L BA	441	57(13)	195(3.4)	0.44	1 (0.5)
A518 (pGA482GG-CP)	A↑, 0mg/L BA	438	71(16)	98 (1.4)	0.22	7 (7.1)
A518 (pGA482GG-CP)	A↑,.5mg/L BA	434	116(27)	335(2.9)	0.77	33(9.9)
A518 (pGA482GG-CP)	A↑, 2mg/L BA	447	130(29)	422(3.2)	0.94	29(6.9)
A518 (pGA482GG-CP)[f]	A↑, 2mg/L BA	417	57 (14)	141(2.5)	0.34	32(22.7)
Sour orange						
EHA101 (pGA482GG-CP)[e]	A↑, 0mg/L BA	852	327(38)	457(1.4)	0.54	11 (2.4)
EHA101 (pGA482GG-CP)[e]	A↑, 1mg/L BA	585	418(71)	604(1.4)	1.03	7 (1.2)
Pineapple sweet orange						
EHA101(pGA482GG-CP)	A↑, 0mg/L BA	442	340(77)	377(1.1)	0.85	9 (2.4)
EHA101(pGA482GG-CP)	A↑, 2mg/L BA	556	335(60)	755(1.4)	1.36	0

[a] A↑ = apical end of the segment protruding from the medium; B↑ = basal end of the segment protruding from the medium; hor = segment placed horizontally on medium; BA = concentration of 6-benzyladenine in selective medium.

[b] GUS+ = shoots that stained positive when assayed histochemically for GUS activity (Jefferson 1987).

[c] Summary of 5 experiments.

[d] CP= coat protein of citrus tristeza virus.

[e] Summary of 2 experiments.

[f] use of 7 ml of liquid kanamycin overlay (100μg/ml).

the budunion and eventually died. Therefore further use of strain A518 was abandoned.

Most shoots that regenerated on selective medium, regardless of the plasmid or Agrostrain used, were not transformed and had "escaped" selection. For instance, data from two experiments in Key lime stem segments using EHA101(pMON9793) produced only three GUS$^+$ shoots out of 551 shoots that regenerated on selection medium (Table 3.1).

The ineffectiveness of kanamycin selection has been reported before for citrus (Kobayashi and Uchimiya 1989, Hidaka *et al.* 1990, Moore *et al.* 1992, Peña *et al.* 1995) and other crops (Dandekar *et al.* 1988, Jordan and McHughen 1988, Serres *et al.* 1992). It has been explained that it is due to the protection of non-transformed cells from the selective agent by surrounding transformed cells, by the cells endogenous non-specific NPTII activity or by persisting *A. tumefaciens* contamination (Dandekar *et al.* 1988, Jordan and McHughen 1988). Others have claimed that escapes were avoided by prolonged continuous exposure of explants to kanamycin, i.e five to six months (Peña *et al.* 1995) or by the addition of a liquid overlay of kanamycin (Serres *et al.* 1992).

Observations of our own experiments showed that shoots regenerate during the first four to five weeks after placing the explants on medium. After three months of exposure to kanamycin in fresh selection medium, no new shoots were obtained and any older shoots bleached and died. Therefore, the addition of kanamycin in a liquid medium overlay was tested to provide a more direct contact of the stem segment explants with the kanamycin. The liquid overlay did not decrease the total number of escape shoots produced compared with equivalent experiments using EHA101(pGA482GG-CP), i.e an average of 3.3 and 3.4 shoots were produced in absence and presence of liquid kanamycin respectively (Table 3.1), and the percentage of GUS+ shoots produced did not increase (1.4 and 0.5). With A518(pGA482GG-CP) the average number of escapes may have slighlty decreased (3.2 to 2.5), while the percentage of GUS$^+$ shoots remained high (22.7). However, use of the overlay was laborious and it induced bacterial overgrowth; moreover strain A518 inhibited rooting of transformed shoots, which developed tumors upon grafting as mentioned above. Thus the use of a kanamycin overlay was not pursued further.

As with Carrizo, the effect of BA concentration (0, 0.5 and 2mg/L) in the medium was evaluated. Absence of BA in the medium reduced the number of shoots produced (escapes) regardless of the Agrostrain and explant used, and in addition the percentage of GUS$^+$ shoots did not decrease. Although fewer shoots regenerated at low BA concentrations, the shoots were larger and rooted more frequently. Data from two experiments with Key lime stem segments, using 0, 0.5 and 2.0 mg/L BA, produced six, seven, and three GUS+ shoots respectively (Table 3.1). One shoot from the experiments with no BA and six from the 0.5 mg/L experiments rooted and remain alive (data not shown) while none of the shoots regenerated from the 2.0 mg/L experiments survive. Key lime node segments produced similar results. Out of 59, 175 and 515 shoots that regenerated in the same concentrations of BA, 3, 13 and 3 GUS$^+$ shoots respectively were obtained (Table 3.2). Seven shoots from the 0.5 mg.L experiments rooted and remain alive.

Sour orange and Pineapple sweet orange. Small experiments with these two cultivars using stem segments and node segments have resulted in the production of transgenic plants. Although few plants have been produced, regenerated sour orange shoots were more vigorous and larger than Key lime and Carrizo shoots. Six GUS+ sour orange plants rooted and survived adaptation in soil.

TABLE 3.2. *Agrobacterium tumefaciens*-mediated transformation using node segments from four citrus cultivars.

Agrobacterium strain and vector plasmid	Treatment [a]	No. node segments cultured	No.(%) segments producing green shoots	Total no. shoots evaluated (avg. per seg. prod. shoots)	Avg. of shoots evaluated per seg. cultured	No.(%) GUS+ [b] shoots
			Carrizo citrange			
EHA101 (pGA482GG-CP)[c]	0.0 mg/L BA	200	38(19)	38(1.0)	(0.19)	3(7.9)
EHA101 (pGA482GG-CP)	1.0 mg/L BA	560	46 (8)	53(1.2)	(0.09)	1(1.9)
			Key lime			
EHA101 (pMON9793)	2.0 mg/L BA	841	460(55)	750(1.6)	(0.89)	0
EHA101 (pGA482GG)	2.0 mg/L BA	286	70 (24)	95(1.4)	(0.33)	0
A518 (pGA482GG)	2.0 mg/L BA	724	198(27)	274(1.4)	(0.38)	4(1.5)
EHA101 (pGA482GG-CP)[d]	0.0 mg/L BA	1205	57 (5)	59(1.0)	(0.05)	3(5.1)
EHA101 (pGA482GG-CP)[d]	0.5 mg/L BA	1117	134(12)	175(1.3)	(0.16)	13(7.4)
EHA101 (pGA482GG-CP)	2.0 mg/L BA	958	364(38)	515(1.4)	(0.54)	3(0.6)
A518 (pGA482GG-CP)	0.0 mg/L BA	390	34 (9)	35(1.0)	(0.09)	1(2.9)
A518 (pGA482GG-CP)	0.5 mg/L BA	392	172(44)	228(1.3)	(0.58)	4(1.8)
A518 (pGA482GG-CP)	2.0 mg/L BA	930	387(42)	397(1.0)	(0.43)	13(3.3)
			Sour orange			
EHA101 (pGA482GG-CP)[d]	0.0 mg/L BA	1273	107 (8)	112(1.0)	(0.09)	0
EHA101 (pGA482GG-CP)[d]	1.0 mg/L BA	874	52 (6)	55(1.1)	(0.06)	0
			Pineapple sweet orange			
EHA101 (pGA482GG-CP)	0.0 mg/L BA	595	175(29)	195(1.1)	(0.33)	2(1.0)
EHA101 (pGA482GG-CP)	2.0 mg/L BA	781	76 (10)	84(1.1)	(0.11)	0

[a] BA = concentration of 6-benzyladenine on selective medium.
[b] GUS+ = shoots that stained positive when assayed histochemically for GUS activity (Jefferson 1987).
[c] CP= coat protein of citrus tristeza virus.
[d] Summary of 2 experiments

Citrus Transformation via Particle Bombardment Followed by *Agrobacterium* Inoculation

This approach was used as a wounding mechanism to enhance *Agrobacterium*-mediated transformation frequencies based on some reports that mention that only cells that have been wounded or traumatized are susceptible

TABLE 3.3. Key lime transformation via particle bombardment followed by *Agrobacterium* inoculation.

Treatment [a]	Number of segments evaluated[b]	No.(%) segments producing green shoots	Total No. shoots evaluated	No.(%) GUS+[c] shoots	No.(%) segments w/GUS+ [c] shoots
		Nodes			
MTMK[d]	642	15 (2.3)	15	2 (13)	2 (0.3)
MTMK 650 psi	81	2 (2)	3	0	0
MTMK 900psi	847	20 (2.3)	22	0	0
MTMK 1100psi	796	24 (3)	26	0	0
MTMK 1550psi	656	5 (0.7)	7	1 (14)	1 (0.1)
MTMK 1800 psi	560	17 (3)	18	2 (11)	2 (0.3)
		Stems			
MTMK	1212	97 (8)	118	12 (10)	11 (0.9)
MTMK 400 psi	90	1 (1.1)	1	0	0
MTMK 900 psi	212	10 (0.5)	14	0	0
MTMK 1100 psi	223	9 (4)	13	0	0
MTMK 1550 psi	284	19 (6.7)	4	0	0
MTMK 1800 psi	135	3 (2)	3	0	0

[a] Treatment = 400 , 650 , 900 , 1100 , 1500 and 1800 psi rupture disk size.
[b] Summary of 2 experiments
[c] GUS+ = shoots that stained positive when assayed histochemically for GUS activity (Jefferson 1987).
[d] MTMK= Selection medium (Murashige and Tucker 1969) containing 0.5 mg/L BA, 200 µg/ml mefoxin and 100 µg/ml kanamycin.

to being transformed by *Agrobacterium* (Hussey *et al.* 1989, Shimoda *et al.* 1990, Bidney *et al.* 1992). In one experiment, wounding nodal explants with a needle was tested based on a report by Hussey *et al.* (1989), who assessed *Agrobacterium*-mediated transformation by stabbing different parts of pea shoots with needles dipped in a suspension of the bacteria. Results of the experiment with key lime nodes produced only two GUS$^+$ shoots out of 1000 total shoots produced. The GUS$^+$ shoots were yellow or bleached and died before any further tests could be done (data not shown). Then we tried bombardment with tungsten-microprojectiles prior to inoculation with *Agrobacterium* (Bidney *et al.*

1992) as a means of inducing a wound response in plant tissues to improve transformation frequencies. We anticipated that this approach would increase transformation especially when using nodal segments as explants. However this did not occur; the total percentage of segments with GUS$^+$ shoots was 0.3% in the MTMK 1800psi experiment, the same as in the control experiment MTMK with no bombardment (Table 3.3). Furthermore transformed shoots regenerated from the bombardment experiment were bleached and fragile.

Evaluation of Transgenic Plants

Polymerase chain reaction. PCR was performed on all regenerated GUS$^+$ shoots to confirm the presence of either the CTV-CP, GUS or NPTII sequences. DNA was extracted from either very small (<50mg fresh weight) leaf pieces from regenerated shoots or from fixed GUS$^+$ stained basal portions of regenerated shoots and subjected to PCR analysis. As mentioned above, shoots regenerated when using vector plasmid A518(pGA482GG-CP) developed tumors and died before any further analysis could be done. However, PCR was performed in most of the Key lime shoots regenerated using fixed GUS$^+$ stained basal portions. DNA was isolated from GUS$^+$ stained fixed stem basal portions, and was amplified with primers specific for the CTV-CP region contained in the T-DNA. A predicted fragment of the CTV-CP gene of about 720 nucleotides was amplified in all DNA samples from GUS$^+$ portions including plasmid DNA used as a positive control (data not shown). The nontransformed citrus control did not yield a product.

Figure 3.2 shows amplification products in some DNA samples from transformed Key lime shoots regenerated from node segments inoculated with EHA101(pGA482GG-CP). DNA was isolated from leaf tissue. The 720 nucleotide fragment amplified in five of the samples, two of which stained entirely in the GUS assay whereas the other three extracted were from chimeric plantlets. The remaining samples where the fragment did not amplify were extracted from chimeric plantlets in which only a tiny sector of the basal sections assayed stained positive for GUS.

Explanations for such variable expression of the GUS gene include chimerism of the regenerated shoots, difficulty of penetration of the X-glucuronide substrate, post-insertional modification of the introduced gene such as methylation, post-translational modification of β-glucuronidase, and variation of the points of gene insertion among the transclones (Serres *et. al* 1992). Thin sectioning revealed that penetration of the substrate was not the major reason for variable GUS expression. We did detect in some transgenic shoots visual evidence, such as sectoring expression, for the presence of chimeric regenerates. However in many other instances we observed random microscopic GUS expression. Most of the plantlets in Figure 3.2 failed to root and died during the adaptation to soil process.

Figure 3.3 shows DNA samples from transformed sour orange, Carrizo citrange and Key lime plants that rooted and grew in soil in a greenhouse. To confirm that the three genes contained within the T-DNA had been transferred, DNA was amplified with primers for CTV-CP, NPTII and GUS genes. A known amount of DNA (6ng/µl) was loaded for all reactions. The amplification of the three fragments in a single PCR reaction was not consistent. Most of the samples showed only two bands: a NPTII fragment of 350bp and a GUS fragment of 450bp. Only a sour orange plant (lane 6) and plasmid DNA used as positive control (lane12) showed all three fragments corresponding to the CTV-CP, NPTII and GUS genes. A nontransformed plant did not amplify any fragment (lane 14) as expected. Lack of amplification of product may be due to insufficient template DNA or primer DNA. Therefore individual PCR reactions were run to confirm the presence of the three sequences. All samples amplified the anticipated size fragments; only a Carrizo plant is shown in Figure 3.4.

Protein blot analysis. Plantlets identified as transgenic by GUS assays and verified by PCR analysis were tested for CTV-CP expression by immunoblot analysis. Preliminary experiments conducted by M. McCaffery in Dr. K. Cline's laboratory with dissected tissue indicated that expression in both transgenic tobacco and Carrizo citrange is high in the petiole. In tobacco, there was a marked enhancement of signal for petiole tissue over that seen for leaf blade. For Carrizo, CP signal was high in both the petiole and leaf blade samples (data not shown). CTV is a phloem-limited virus; therefore, tissue samples for the immunoblot assays consisted of bark, petiole and midribs from transformed plants.

Figure 3.5 shows Protein analysis results of six sour orange shoots regenerated from stem segments, three Carrizo citrange shoots (one shoot regenerated from a node segment and the others from stem segments), and two key lime shoots regenerated from node segments. Samples were incubated with polyclonal antibody 894. Only two sour orange plants and one Carrizo plant expressed the protein. It is important to mention that the two sour orange plants had stained entirely blue during the GUS assay while the Carrizo plant was chimeric in its expression; however a large sector of the cross section stained positive. Protein expression in these plants was in comparable levels to the CTV infected material. The remaining plants in this Protein blot were chimeric in their expression of GUS and even though one of the Key lime plants had amplified a PCR band for the GUS gene (lane 13 in Figure 3.5 is same plant as lane 9 in Figure 3.3), all of these plants failed to show the protein in several immunoblots performed. This lack of protein expression may be because when these plants were assayed for GUS activity, only a very tiny sector of the cross section stained and so the majority of cells in the regenerated plants may not be transgenic. Extracting the samples in a 1:1 (w/v) ratio instead of 1: 4 (w/v) of sampling buffer did not change the results (data not shown).

Figure 3.6 shows Protein analysis results of two Carrizo citrange shoots and two sour orange shoots regenerated from stem segments, and one Carrizo citrange shoot regenerated from a node segment. Monoclonal antibody MCA13 was used in this blot. Equal amounts of samples were loaded (ratio of 1:4 [w/v] of SDS-PAGE sample buffer). Samples from two Carrizo shoots were extracted twice with different extraction methods. One consisted of grinding the sample in a 1.5 ml eppendorf tube with a minipestle in the presence of liquid nitrogen. In the second method, plant sap was extracted by pressing the tissue using an electrical rotor (MAXI-TORK^{TM} 42522, Dayton Electric Co. Chicago 60648, USA). Most samples extracted with the first extraction method (lanes 2,4,5,6 and 8), expressed the CP in comparable levels, except a Carrizo plant in lane 3 that expressed a lower level. In any case, expression was in lower levels compared to that found in T36 infected Etrog citron tissue (lane 11). One of the Carrizo samples did not exhibit CP (lane 7) when extracted with the electrical rotor. This is the same Carrizo plantlet that expressed poorly in lane 3. Probably degradation of the protein occured during the extraction, due to lack of liquid nitrogen. A nontransformed Carrizo citrange plantlet did not express the CP.

Figure 3.7 shows Protein analysis results of the same transgenic plantlets when incubated with polyclonal antibody 894. Equal amounts of samples were extracted by grinding the tissue in a 1.5 ml eppendorf tube with a minipestle in the presence of liquid nitrogen. All samples showed comparable levels of CP (lanes 1 through 5); however, lower levels than those found in T36-infected Etrog citron tissue (lane 8), T36-infected Madam Vinous sweet orange (lane 9), T-36 infected Key lime tissue (lane 10) and T-64 infected *C. excelsa* tissue (lane 11). No CP was seen in the nontransformed control (lane 6).

Immunoblot analysis of CTV infected plant material shows three bands (lanes 2 and 14 in Figure 3.5, lane 11 in Figure 3.5 and lanes 8 through 11 in Figure 3.7). According to Sekiya *et al.* (1991) these bands correspond to the intact CP, CP1 and CP2 of M_r 25 Kd, 24 Kd and 21Kd respectively, the 25 Kd CP and 21 Kd CP2 being more pronounced than the 24 Kd CP1. They suggested that CP1 and CP2 originate as specific hydrolysis products of the intact CP, probably by post-translational proteolysis. More specifically, they stated that CP1 is the result of specific cleavage of the intact CP between 11 and 15 amino acids of the amino-terminus, and CP2 is the result of hydrolysis of intact CP at amino acid 26. These three bands are barely visible in one Carrizo transgenic plant (lane 8 in Figure 3.6) whereas two bands corresponding to the full length CP and CP1 are slightly seen in lanes 2 and 6 of Figure 3.5 (plant in lane 2 is the same Carrizo plant as in lane 8). In most transgenic plants tested by immunoblot only one band corresponding to the intact CP can be detected (Figure 3.7). Since the two degradation products arise from the CP by the action of proteolytic enzymes, we can only speculate that these proteases, either host-specific as suggested by Pappu *et al.* (1994, 1995) or viral-specific, are either present in too low amounts to produce proteolysis or are non-existent in the transgenic plants. It is also possible that in the transgenic plants, CP is not properly folded and that the amino-terminus is not exposed to proteases. One more possibility is that, considering that there seems to be a very low amount of CP in the transgenic plants to start with compared with the CTV infected controls, even if the proteases are present and degrading the CP, a very low quantity of degradation products is formed, making them barely detectable.

DNA blot analysis. DNA was extracted from approximately one gram of fresh bark and leaf tissue using a modified Dellaporta *et al.* (1983) procedure (Durham 1990). Samples included two transformed sour oranges plants regenerated from stem segments, one transformed Carrizo plant regenerated from a node segment and one non-transformed sour orange plant as a control. Samples were restricted either with *KpnI* or *HpaI*, to produce an internal or border fragment respectively. The probe was a 669bp *NcoI* → *NcoI* fragment containing the CTV-CP gene isolated from plasmid pGA482GG-CTVCP. No bands were obtained when nontransformed sour orange DNA was hybridized with the probe, lanes 6 and 10 (Figure 3.8).

Digestion of plant DNA with *KpnI* yielded a single band of ≈ 1.0 kb in all samples, lanes 3 through 5 (Figure 3.8). The bands seem to be of equal intensity in both sour orange samples, however the band is more intense in the Carrizo DNA. This band corresponds to an internal fragment of ≈ 957 bp produced by the presence of recognition sites of *KpnI* within the T-DNA region of plasmid pGA482GG-CTVCP, in CaMV 35S promoter cassette which includes the CTV-CP. As the T-DNA from pGA482GG-CP contains only one recognition site for *HpaI* (Figure 3.1), digestion of plant DNA with this enzyme should yield hybridizing fragments that are composed of both T-DNA and flanking citrus DNA sequences, so that they all represent border fragments. The length of these fragments should vary, depending on the location of the nearest *HpaI* site flanking the integrated T-DNA. Digestion of DNA from these plants with *HpaI* yielded bands of variable size, and in all cases, the bands were of a larger size than the expected minimum border fragment (≈ 4.5 kb). As shown in Figure 3.8, only one hybridization band is detectable in the two sour orange plants, the first sour orange plant (lane 7) yielded a band of ≈ 6.0 kb, while the second sour orange plant yielded a band of ≈4 - 5.0 kb (lane 8). The remaining plant, a

Carrizo, contains two hybridizing fragments one of ≈ 6.0 kb and a second one of ≈ 9.0 kb (lane 9). The number of T-DNA inserts was expected to equal the number of bands detected; consequently, both transgenic sour orange plants appear to have one single T-DNA insert, whereas the Carrizo plant may have two inserts.

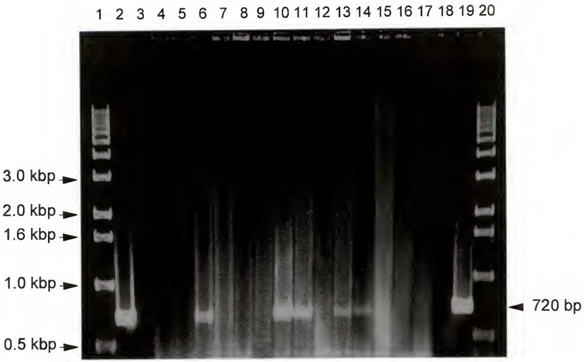

Figure 3.2. Polymerase chain reaction analysis to detect the presence of the CTV-CP gene in transgenic Key lime plantlets regenerated from node segments inoculated with EHA101(pGA482GG-CP). DNA was isolated from GUS$^+$ and chimeric green shoot leaf tissue. The 720bp PCR product was visualized on an 1.5% agarose-ethidium bromide gel. Lanes 1 and 20, 1 kb ladder molecular weight markers; lanes 2 and 19, plasmid DNA from pGA482GG-CP; lanes 3 and 18, empty; lanes 4-5 and 12, nontransformed key lime control plantlets; lanes 6-11,15-16, DNA from 8 chimeric plantlets; lanes 13 and 14, DNA from two solidly GUS$^+$ plantlets. The anticipated size fragment was amplified in the two solidly GUS$^+$ plantlets and in three chimeric plantlets. None of the negative controls yielded a product.

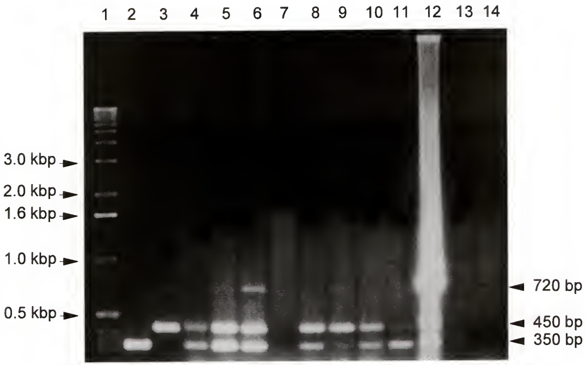

Figure 3.3. Polymerase chain reaction analysis to detect the presence of the CTV-CP, NPTII and GUS genes in transgenic sour orange, Carrizo and Key lime plants regenerated from stem and node segments inoculated with EHA101(pGA482GG-CP). DNA was extracted from solidly and chimeric GUS$^+$ leaf tissue and amplified with primers for the three genes in a single reaction (except in lanes 2 and 3). PCR products of 350bp (NPTII), 450bp (GUS) and 720bp (CTV-CP) were visualized on an 1.5% agarose-ethidium bromide gel. Lane 1, 1 kb ladder molecular weight marker; lanes 2 and 3, plasmid DNA from pMON9793 amplified with NPTII primers and GUS primers respectively; lanes 4 and 6, DNA from two solidly GUS$^+$ sour orange shoots regenerated from stem segments; lane 5, DNA from a chimeric Carrizo shoot regenerated from a node segment; lanes 7 through 10, DNA from four chimeric Key lime shoots regenerated from node segments; lane 11, DNA from a chimeric Key lime shoot regenerated from a stem segment inoculated with EHA101(pMON9793); lane 12, plasmid DNA amplified with CTV-CP, NPTII and GUS primers; lane 13, empty; lane 14, DNA from a nontransformed Key lime plantlet. Most of the samples showed two bands: an NPTII fragment of 350bp and a GUS fragment of 450bp. Only a sour orange plant (lane 6) and plasmid DNA used as positive control (lane12) showed three fragments corresponding to CTV-CP, NPTII and GUS genes. A nontransformed plant did not amplify any fragment (lane 14) as expected. Lack of amplification of the three products may be due to either insufficient template DNA or primer DNA.

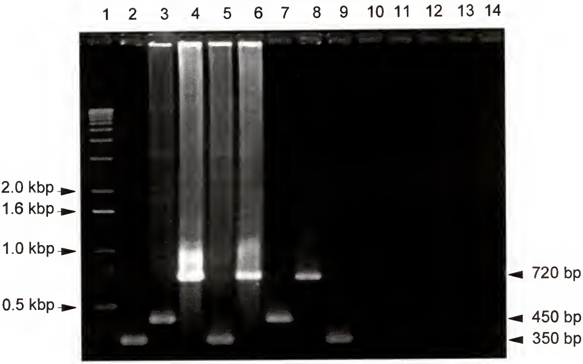

Figure 3.4. Polymerase chain reaction analysis to detect the presence of CTV-CP, NPTII and GUS genes in a transgenic Carrizo plantlet regenerated from a node segment inoculated with EHA101(pGA482GG-CP). DNA was isolated from a chimeric GUS$^+$ leaf tissue and amplified with one set of primers per reaction. PCR products of 350bp (NPTII), 450bp (GUS) and 720bp (CTV-CP) were visualized on an 1.5% agarose-ethidium bromide gel. Lane 1, 1 kb ladder molecular weight marker; lane 2, plasmid DNA from EHA101(pMON9793) amplified with NPTII primer; lanes 3 through 5, plasmid DNA from EHA101(pGA482GG-CP) amplified with GUS, CTV-CP and NPTII primers respectively; lane 6, plasmid DNA from EHA101(pGA482GG-CP) amplified with the three primers; lanes 7 through 9, DNA from one chimeric Carrizo shoot amplified with GUS, CTV-CP, NPTII primers respectively; lane 10, DNA from the same Carrizo shoot amplified with the three primers; lanes 11 through 13, DNA from one nontransformed sour orange plantlet amplified with GUS, CTV-CP,and NPTII primers respectively; lane 14, DNA from the same nontransformed sour orange amplified with the three primers. The anticipated size fragments were visualized in all individual PCR reactions (Carrizo DNA and plasmid DNA), however we failed to amplify the three fragments in lanes 6 and 10. Nontransformed control did not yield a product.

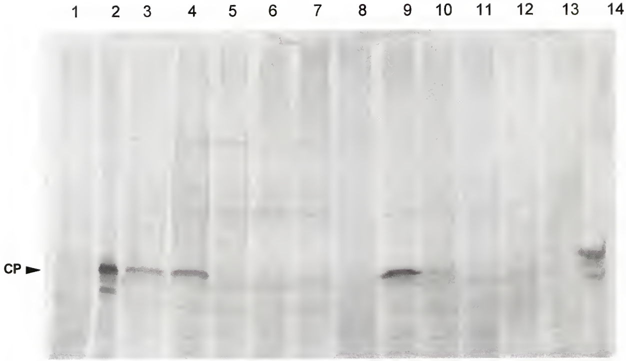

Figure 3.5. Protein analysis of PCR verified transgenic citrus plantlets with polyclonal 894 anti-CTV CP serum. Tissue samples consisted of bark, petiole and midribs from transformed plants growing in a greenhouse. SDS-PAGE sample buffer (10% mercaptoethanol, 20% glycerol, 50mM Tris, 0.5% SDS) was added to the sample at a ratio of 1:4 (w/v). Lane 1, tissue from a nontransformed sour orange plant; lane 2, T36-infected Etrog citron tissue; lanes 3 and 4, tissue from two putatively transformed sour orange plants; lanes 5 to 8, tissue from putative transgenic chimeric sour orange plants; lane 9, tissue from a putative chimeric transgenic Carrizo plant regenerated from a node segment; lanes 10 and 11, tissue from putatively transformed chimeric Carrizo plants; lanes 12 and 13, tissue from two putative transgenic chimeric key lime plants; lane 14, T36-infected Etrog citron tissue.

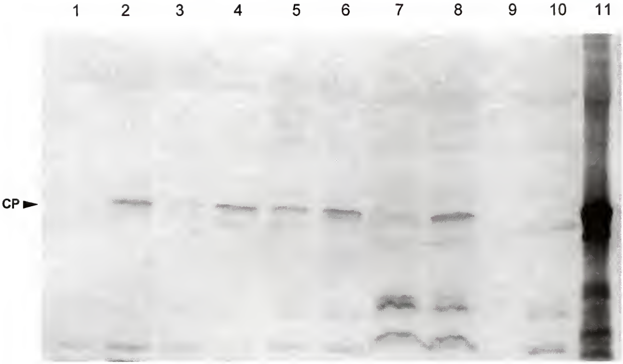

Figure 3.6. Protein analysis of PCR verified transgenic citrus plantlets with monoclonal MCA13 anti-CTV CP serum. Tissue samples consisted of bark, petiole and midribs from transformed plants growing in a greenhouse. SDS-PAGE sample buffer (10% mercaptoethanol, 20% glycerol, 50mM Tris, 0.5% SDS) was added to the sample at a ratio of 1:4 (w/v). Lane 1, uninfected Carrizo citrange tissue; lane 2, tissue from a Carrizo plant putatively transformed with a CTV CP sense construct; lane 3, tissue from a second Carrizo plant; lane 4, tissue from a putatively transformed sour orange plant; lane 5, tissue from a second putative transgenic sour orange plant; lane 6, tissue from a third putative transgenic Carrizo plant regenerated from a node segment; lanes 7 and 8, tissue from the same Carrizo plants in lanes 2 and 3 but from a different extraction procedure; lane 9, 1x SDS buffer; lane 10, nontransformed Carrizo citrange tissue; Lane 11, T36 infected Etrog citron tissue.

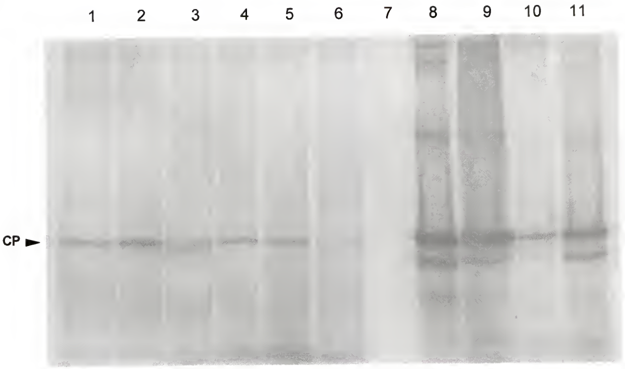

Figure 3.7. Protein analysis of PCR verified transgenic citrus plantlets with polyclonal 894 anti-CTV CP serum. Tissue samples consisted of bark, petiole and midribs from transformed plants growing in a greenhouse. SDS-PAGE sample buffer (10% mercaptoethanol, 20% glycerol, 50mM Tris, 0.5% SDS) was added to the sample at a ratio of 1:4 (w/v). Lane 1, tissue from a putatively transformed sour orange plant; lane 2, tissue from a second putative transgenic sour orange plant; lane 3, tissue from a putative transgenic Carrizo plant regenerated from a node segment; lane 4, tissue from a second putatively transformed Carrizo plant; lane 5, tissue from a third putative transgenic Carrizo plant; lane 6, tissue from a non transformed sour orange plant; lane 7, 1x SDS buffer; lane 8, tissue from T-36 infected key lime; lane 9, T36-infected Etrog citron tissue; lane 10, T36-infected Madam Vinous sweet orange; lane 11, T-64 infected *C. excelsa* tissue.

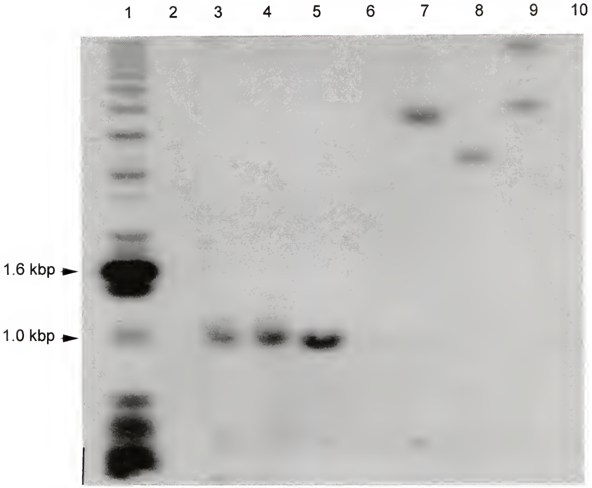

Figure 3.8. DNA analysis of two sour orange plants regenerated from stem segments and one Carrizo citrange plant regenerated from a node segment inoculated with EHA101(pGA482GG-CP). The probe was a 669bp *NcoI* → *NcoI* fragment containing the CTV-CP gene isolated from plasmid pGA482GG-CTVCP. Lane 1, 1 kb ladder molecular weight marker; lane 2, empty; lane 3, sour orange DNA cut with KpnI; lane 4, DNA from a second sour orange plant cut with KpnI; lane 5, Carrizo citrange DNA cut with KpnI; lane 6, nontransformed sour orange plant cut with KpnI; lane 7, DNA from same sour orange in lane 3 cut with *HpaI*; lane 8, DNA from the same sour orange in lane 4 cut with *HpaI*; lane 9, DNA from the same carrizo Citrange plant in lane 5 cut with *HpaI*; lane 10, non-transformed sour orange plant DNA cut with *HpaI*.

CHAPTER 4
CHALLENGE INOCULATION OF TRANSFORMED CITRUS PLANTS

Introduction

CTV is found in most citrus growing areas of the world and is economically the most important citrus virus worldwide. It is responsible for quick decline, stem pitting, vein-clearing, lime disease and seedling yellows (Bar-Joseph *et al.* 1979) the most dramatic of which is quick decline. CTV is a phloem-limited, flexuous closterovirus approximately 2,000 x 11 nm in size, which is aphid transmitted in a semi-persistent manner (Bar-Joseph and Lee 1980). The virus can be transmitted by grafting and mechanically by slash inoculating partially purified virus into the stem of a receptor citrus host (Garnsey *et al.* 1977). The best approaches for CTV control are avoidance of the pathogen, selection of cultivars which exhibit virus resistance and use of certified virus-free propagating material (Agrios 1988). Another strategy that has successfully prevented CTV tree loss is cross protection (Muller and Costa 1977, Costa and Muller 1980, Gonsalves and Garnsey 1989). Cross protection involves infecting a plant with a mild isolate of a virus in order to prevent disease development due to infection by severe strains (Gonsalves and Garnsey 1989).

In the last decade transgenic plants carrying nucleotide sequences derived from plant viruses have shown different levels of resistance to infection or suppression of disease symptoms. In 1986, Powell-Abel *et al.* were the first to demonstrate that transgenic tobacco expressing the coat protein (CP) gene of tobacco mosaic virus (TMV) was resistant to TMV infection. As expected, many of these initial experiments were performed with tobacco because of it is susceptible to numerous plant viruses and is easily transformed by *Agrobacterium tumefaciens.* Many other reports (reviewed above) have since shown that this phenomenon is broadly applicable to different groups of plant viruses and commercially important crops.

Thus, this represents a generic method to produce virus resistance. The phenomenon was given the name CP-mediated resistance. Transgenic plants that express virus CP are otherwise indistinguishable form the parent plants. This resistance mimicks cross protection in a number of ways (Beachy *et al.* 1987)

The objectives of this study were to evaluate the cross protecting ability of transgenic sour orange citrus plants expressing the CP of CTV when challenged with the severe strain T-36 or the isolate T-64.

Materials and Methods

Plant Material

Two transgenic sour orange [*C. aurantium* L.] plants, identified as 5/1/1 and 8/1/1, obtained from the transformation experiments, were used to evaluate CP-mediated protection against CTV infection. Expression of the CTV-CP gene, in these plants was confirmed by PCR, DNA blot and Protein blot analysis. They were multiplied by graft propagation either on rough lemon (*C. jambhiri* Lush.) or on sour orange rootstock, in order to increase the number of plants for challenge inoculation experiments. Additionally, four non-transformed sour orange plants were grafted onto rough lemon and three were grafted onto sour orange. These plants were used as controls.

All grafted-transgenic plants (transclones) and the seven controls were tested histochemically for GUS enzyme activity. All transclones stained totally blue (positive reaction). The non-transformed controls did not stain blue.

Plants were grown in a commercial potting mixture (growing medium, MetroMix 500 from Grace Sierra) in two liter plastic containers, and fertilized with a mixture of NPK (20-20-20) every month. After six months, the formula was changed to NPK (20-2-10). Pest and disease management included the periodic application of Talstar, Orthene and Pentac three times at weekly intervals, followed by one application of Enstar II. The experiment was conducted in a greenhouse with mean minimum and maximum temperatures of 21 and 28°C, respectively.

Challenge Inoculation Experiments

Experiment one. A total of 12 bud propagation of transgenic clones (from the 5/1/1 plant) grafted as scions onto rough lemon rootstock were grafted again, with non-transformed Madam Vinous sweet orange so that the transgenic sour orange formed an interstock between the rough lemon and the sweet orange (Figure 4.1A). This was done to allow us to test for the CTV-induced decline reaction that isolate T-36 causes at the budunion of sweet orange grafted on sour orange. Two control plants, where the interstock was a non-transgenic sour orange, were included in this experiment.

Experiment two. Another 12 rough lemon rootstocks were grafted with two buds, one of non-transformed sour orange and one of a transformed sour orange (eight coming from the 5/1/1 plant and four from the 8/1/1 plant), so that one branch would be a transgenic sour orange and the other a non-transgenic sour orange (Figure 4.1B).

Experiment three. Twenty nine transclones (22 from the 5/1/1 plant and seven from the 8/1/1 plant) propagated onto sour orange rootstock were used in this experiment (Figure 4.1C). Three control plants, that is a non-transgenic sour orange grafted onto sour orange, were included in the experiment.

All tissue sources used as rootstocks, interstocks and scions, were evaluated by serological indexing (see below) to confirm the absence of CTV before they were challenge inoculated.

Virus Isolates and Donor Hosts

Two Florida CTV isolates, kindly provided by Dr. Steve Garnsey (USDA-ARS Orlando), were used. Isolate T-36 is a severe strain from Florida which was originally collected from a sweet orange tree on sour orange rootstock suffering from quick decline (Garnsey and Jackson, 1975) and produces severe symptoms on Mexican lime, a decline in sweet orange-sour orange trees, and a mild seedling yellows reaction. T-36 is also the isolate from which the CP construct used in the transformation experiments was derived. Isolate T-64 produces epinasty in sour orange (personal communication with Dr. Garnsey). Isolate T-36 was propagated in Madam Vinous sweet orange and T-64 in *C. excelsa*. Plants were maintained in a greenhouse with mean minimum and maximum temperatures of 21 and 28 °C, respectively. Inoculum source tissue from donor hosts was evaluated by serological indexing (see below) to confirm the presence of CTV before being used as inoculum.

Challenge Inoculation with CTV

Rectangular bark pieces and rectangular leaf pieces, including the midrib, from T-36 and T-64 donor plants were used to inoculate the transclones. Plants

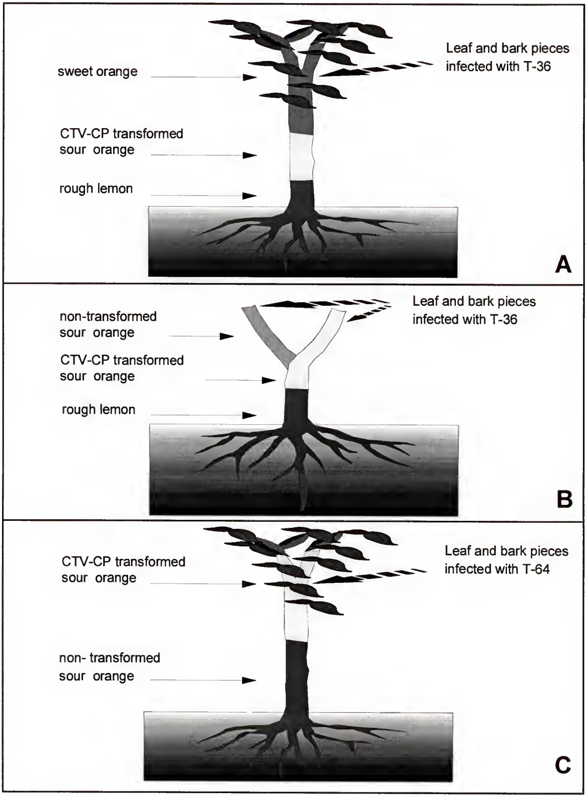
sweet orange
Leaf and bark pieces infected with T-36
CTV-CP transformed sour orange
rough lemon
A
non-transformed sour orange
Leaf and bark pieces infected with T-36
CTV-CP transformed sour orange
rough lemon
B
CTV-CP transformed sour orange
Leaf and bark pieces infected with T-64
non- transformed sour orange
C

Figure 4.1. Rootstock / scion combinations and CTV isolates used for coat protein-mediated protection evaluation. A) experiment 1; B) experiment 2; C) experiment 3.

from experiment one were inoculated in the stem of the sweet orange scion with T-36 isolate (figure 4.1 A). Plants from experiment two were inoculated with T36 on each branch, the transgenic sour orange and the non-transgenic sour orange (Figure 4.1 B). Plants from experiment three were inoculated in the stem of the transgenic sour orange with isolate T-64 (Figure 4.1 C).

In all experiments, two pieces of inoculum per plant were used, one bark piece and one leaf piece. Inoculum tissue was sealed firmly into the receptor stems with plastic grafting tape. Three weeks later the grafting tape was removed and the plants were evaluated for survival of the grafted inoculum tissue. All inoculations were first done between 7/14/94 and 7/18/94. Plants were reinoculated if the grafted tissue did not survive, as happened in experiments two and three. These were reinoculated twice (on 9/20 and 10/21). Surviving inoculum tissue was left in place for the duration of the experiment.

Serological Tests

CP-mediated protection was evaluated by detecting the replication or the lack of replication of CTV isolates in the inoculated plants. Enzyme-linked immunosorbent assays (ELISA) were conducted to determine presence/absence of the virus. Double antibody sandwich-indirect (DAS-I) was performed as described by Garnsey and Cambra (1991). Polyclonal antiserum number 894 (kindly provided by Dr. D. Purcifull) prepared to sodium dodecyl sulfate (SDS) degraded CTV T-4 CP was used as the source of coating antibody (Garnsey *et al.* 1979). Gamma globulins (IgG) were separated from the whole serum by the protein A-Sepharose affinity chromatography procedure of Miller and Stone (1978). The concentration of IgG was estimated spectrophotometrically using E 0.1%/280nm = 1.4. Ascites fluid containing the monoclonal antibody MCA13 (provided by Dr. S. Garnsey) produced to T-36 was used as source of the intermediate antibody (Permar *et al.* 1990). Commercial goat anti-mouse IgG (Boehringer Mannheim Biochemicals) labeled with alkaline phosphatase was used.

Polystyrene Immulon IV microtiter plates (Dynatech Laboratories) were coated with 2µg/ml of purified IgG of antibody 894 in carbonate buffer (0.015 M $NaHCO_3$, 0.03 M $NaCO_3$, pH 9.6) and incubated for 15-18 hrs at 4°C. A volume of 200µl per well was used. Antigen samples were extracted in extraction buffer [phosphate-buffered saline containing 0.5 percent Tween 20 (PBST) + 2% polyvinyl pyrrolidone (PVP) + 0.5% bovine serum albumen (BSA) buffer] at a 1: 20 dilution (w/v). Next ten fold dilutions of 1:200 and 1:2000 were prepared in extraction buffer. In the last two sampling dates a dilution of 1:5 was used. After washing plates with PBST, samples were added to the wells and incubated for 2 hrs at 37°C. Following washing, MCA13 was added at a dilution of 1:4,000 (v/v) in conjugate buffer [phosphate-buffered saline (PBS) + 0.5% BSA] and plates incubated for 2 hrs at 37°C. After washing, goat anti-mouse IgG labeled with alkaline phosphatase at a dilution of 1:5,000 in conjugate buffer (PBST + 0.5% BSA) was added and incubated for 2 hrs at 37°C. The plates were washed and substrate added (one mg/ml of p-nitrophenyl phosphate (Sigma) in 10% triethanolamine, pH 9.8). The reaction was measured after 60 min and 120 min at 405 nm (OD_{405}) with a microtiter plate reader (MICROPLATE EL309, Bio-tek instruments). Samples were considered positive when OD_{405nm} values were three times the mean of healthy controls.

For all serological tests, two replications were used per sample. Positive controls included non-transgenic plants inoculated with isolates T-36 and T-64. Negative controls included extraction buffer and similar buffer extracts from healthy Madam Vinous sweet orange and non-inoculated transformed sour orange.

To detect the expression of the transgenic CP in the transclones we tested different combinations of various monoclonal and polyclonal antibodies (kindly provided by Dr. R.F. Lee and Mr. K.L. Manjunath). Four monoclonal antibodies in addition to MCA13 and three polyclonal antibodies in addition to 894 were used. Monoclonal antibodies were used at a 1:5000 dilution and polyclonal antibodies at 1:1000. CTV MCA13 is a monoclonal antibody (MAB) which reacts with severe sources of CTV, but does not react to mild isolates from Florida and some other countries (Permar et al. 1990). The 3DF1 and 3CA5 MABs are from Spain and react with most isolates of CTV (Vela *et al.* 1988). They are specific to two separate and widely conserved epitopes on the CTV CP (Garnsey *et al.* 1989). Monoclonal F10 was produced in Cuba against a Cuban CTV isolate (K.L. Manjunath, personal communication). MAB 17G11 was produced in Florida by M.E. Hooker and A.C. Powell to a mixed antigen (K.L. Manjunath, personal communication). Polyclonal antibody (PAB) number 1053 was prepared against whole, unfixed CTV isolate T26 (Rocha-Peña *et al.* 1991). PAB 894 was prepared to SDS degraded CTV isolate T4 (Garnsey *et al.* 1979), and antiserum 879 was prepared to undegraded whole unfixed CTV isolate T4 (Brlansky *et al.* 1984). PAB CREC 35-4 was produced against the Indian isolate B227, which causes stem pitting in mandarins (*Citrus reticulata* Blanco) and rough lemon (Manjunath et al. 1992).

Sampling

Antigen samples consisting of bark, petiole or midribs from the transformed sour orange interstock or branch were collected every month for serological tests. Individually wrapped sterile blades were used to collect samples from each inoculated and non-inoculated (control) plant. Sistemic infection of CTV in inoculated plants of the size used requires from two to six months (Dr. S. Garnsey personal communication); therefore extraction of samples was done on bulked tissue from individual plants per experiment during the first months. Once we detected positive results by ELISA tests, samples were extracted individually to identify the plants where the virus was replicating. We also sampled other plant parts such as the rough lemon rootstock and the sweet orange scion to see if the virus moved into these parts.

Results and Discussion

For the challenge experiments we decided to evaluate CP-mediated protection by detecting the replication or the lack of replication of CTV isolates in the inoculated plants. For this purpose we first conducted DAS-I ELISA assays to determine the presence of the CTV CP in the two original transgenic sour orange plants with the goal of establishing an OD reading range that would allow us to differentiate between the expression of the transgenic CP and the replication of the virus after inoculation. We used polyclonal antibody 894 as coating antibody and monoclonal MCA13 as the intermediate antibody for the assay as Dr. Garnsey does routinely to detect CTV.

To our surprise, we were not able to detect the CP in the transgenic plants even at a 1:1 dilution of the extracted sample whereas in the donor T-36 infected plant we detected the virus at a 1:2000 dilution (data not shown). At this point, it seemed that transgenic plants apparently did not produce detectable levels of CP for ELISA tests.

We then proceeded to test different combinations of various monoclonal and polyclonal antibodies. For this test five monoclonal antibodies and four polyclonal antibodies were used (see above). We found that by using the monoclonal antibody 3DF1 in combination with any of the polyclonal antibodies we could detect the presence of the CTV-CP in transgenic sour orange and Carrizo plants (Table 4.1 shows results in sour orange 5/1/1). The ELISA readings were higher when using PABs 1053 and CREC 35-4 as coating antibody than when using antibodies 894 or 879. Monoclonal antibody 3CA5 also detected the CP in combination with two polyclonal antibodies: CREC 35-4 and 879. CP was detected in virus (T-36) infected donor plants with all monoclonal and polyclonal antibodies tested (data not shown).

TABLE 4.1. DAS-I ELISA[a] test of various monoclonal and polyclonal antibodies to detect the presence of citrus tristeza virus CP in one transgenic sour orange plant (5/1/1).

	Polyclonal antibodies			
Monoclonal Antibodies	**1053**	**CREC 35-4**	**894**	**879**
F10	-	-	-	-
17G11	-	-	-	-
3DF1	++	++	+	+
3CA5	-	+	-	+
MCA13	-	-	-	-

[a] DAS-I = Double antibody sandwich-indirect ELISA = enzyme-linked immunosorbent assay. Samples were extracted at 1:20 dilution. Presence of CTV-CP was determined by ELISA at OD $_{405nm}$. (-) is 0.007,(+) is 0.014 to 0.025, (++) is 0.026 to 0.060.

Polyclonal antibodies (PABs) in general comprise a number of different antibodies which have been produced against the epitopes present in the antigen injected into the immunized animal species. Therefore, they are able to react with a wide range of strains or variants of the virus sharing common epitopes. In contrast, monoclonal antibodies (MABs) produced by hybridoma technology are specific to a single antigen epitope. They react only to those strains of the virus that share the antibody-specific epitope. MABs have greater potential for strain differentiation (Rocha-Peña 1990, Pappu *et al.* 1994b).

We believe that the detection or lack of detection of the CP in transgenic plants may be due to one or more reasons, serological and biological. First, it may be due to the different specific recognition sites of the monoclonal antibodies within the CP. It has been reported that a single amino acid at position 124 of the CP constitutes the MCA13 reactive epitope (Pappu *et al.* 1992). It was later shown that the amino acid phenylalanine (Phe) at position 124 of the CP is critical for the binding of MCA13 (Pappu *et al.* 1994) and that this amino acid was present in all the severe, MCA13 reactive strains, while it was replaced by a tyrosine (Tyr) in all the mild, MCA13 nonreactive strains Pappu *et al.* 1993). While sequences in the middle of the CP are responsible for the specificity of MCA13, the extreme amino terminus of the CP consitutes the antigenic determinant for 3DF1 and 3CA5 as reported by Sekiya et al. (1991) and Vela *et al.* (1988) and Garnsey *et al.* (1989) respectively. More recently it was demostrated that the amino acid aspartic acid (Asp) at position 2 of the intact CP of CTV contains the epitope for MAB 3DF1 (Pappu *et al.* 1994, 1995). Pappu *et al.* presented evidence that a single amino acid change in the CP can eliminate or drastically alter an MABs ability to detect a given strain of CTV. Serological studies with additional MABs showed that CTV contains other epitopes (Garnsey *et al.* 1989, Tsai and Su 1991).

Second, depending on the extraction procedure of the samples, denatured versus nondenatured as in Protein blots and ELISA assays respectively, different epitopes critical for detection with different MABs may be or may not be available for exposure causing various results in serological detection. It has been recommended that a combination of MABs or PABs that recognize different epitopes on the CTV CP be used for such important applications as CTV indexing and certification programs (Cambra *et al.* 1990).

Third, PABs can be produced against whole unfixed virus, against whole fixed virus, as well as against degraded CP of the virus, all of which may produce contrasting results in serological tests. However, the broad specificity of PABs make it very unlikely that they could drastically alter a result as MABs could.

Fourth, it has been reported that, in general, procedures where the antigen is trapped to a solid phase (immunoblots and plate trapped antigen ELISA) are less sensitive for detection of viruses in plant extracts than procedures where the antigen is trapped by an antibody bound to the solid phase as in sandwich forms of ELISA (Garnsey *et al.* 1993). In antigen trapped assays there is competitive binding of host protein and antigens in the extract to the solid phase and when the virus titer is low there may be insufficient binding of the pathogen-specific antigen. In Protein blots, however, the protein bands should be relatively pure if properly separated and the signal is concentrated.

All or any one of these possibilities may explain why we were able to detect the presence of the CTV coat-protein in Protein blots of transgenic sour orange plants using MAB MCA13 and PAB 894 (Plates 3.5 to 3.7) while we failed to detect it in ELISA tests using the same antibodies. In the immunoblots, samples were SDS degraded during the extraction procedure, then subjected to polyacrilamide gel electrophoresis in order to separate the various proteins before blotting and incubating the samples with the antibodies. Therefore the denaturation of the CP may have made the critical amino acid for MCA13 reactivity at position 124, available for detection, whereas in ELISA assays in which the protein was not subjected to denaturation, this epitope was unavailable for detection in the CP produced by transgenic plants. The fact that ELISA assays detected viral CP in CTV infected control plants but did not detect CP produced by transgenic plants may suggest that, the CP gene transferred to the transgenic plants has been somehow rearranged such that the availability of different epitopes has changed. It is also possible that incubation of the samples during 2 hrs at 37°C in ELISA assays, may have resulted in total proteolysis of the transgenic CP. Another possibility is that some changes may have been induced during tissue culture regeneration of citrus plantlets. However futher analysis need to be done to evaluate these considerations. The fact that MAB 3DF1 could detect the CP in transgenic plants in ELISA assays may be reasonable if we consider that, since the most critical amino acid for reactivity is at position 2 of the CP, the extreme location of this epitope may allow it to stick out, making it available for detection even if the protein is not denatured.

Lack of detection of the CP of other viruses in transgenic plants using ELISA assays has been reported before. Gonsalves *et al.* (1994) obtained transgenic muskmelon plants containing Cucumber Mosaic Virus-White Leaf Strain CP gene(CMV WL CP). PCR results showed that 21 of the 23 putative transgenic lines characterized contained the GUS, NPTII and CMV WL CP genes. ELISA assays were performed to detect both CP (CP ELISA) and NPTII (NPTII ELISA) gene expression. Nine of the transgenic lines were negative in the CP ELISA and only two of these were positive for NPTII ELISA. Protein blot analysis of these nine plants was either negative or not tested. The authors did not discuss possible explanations.

We decided to use MCA13 and 894 antiserum in DAS-I ELISA tests to detect the replication or lack of replication of virus in challenged plants for several reasons; one, since no CP of transgenic plants could be detected with this combination, we could assume that a positive reading in ELISA would be evidence of replication of the challenge virus itself, second, DAS-I is the standard assay used by others, for sensitive detection of CTV (Garnsey and Cambra, 1991), and third, these two antisera were produced in Florida and were readily available to us during the experiment.

Nine weeks after inoculation, bulked samples of plants in experiment one (Figure 4.1.A) gave positive ELISA readings for the presence of CTV. Therefore subsequent tests were done on individually extracted samples. None of the plants in the other two experiments showed any indication of replication of virus. However, in these plants the inoculum tissue piece looked dry or dead. Thus re-inoculations were done. As mentioned in material and methods, experiment one consisted of transclones from the 5/1/1 plant while experiments two and three included transclones from both 5/1/1 and 8/1/1 plants. This was due to problems during the propagation grafting process where we failed to successfully graft propagate enough 5/1/1 transclones.

ELISA tests of individual plants in experiment one at 63 and 100 days after inoculation (dai.) produced positive results in only one plant, number 4 (Table 4.2). The CTV antigen was detectable at a 1:20 dilution of tissue extract, similar to the non-transformed inoculated control (plant 56). In contrast the virus antigen could be detected at a 1:2000 dilution of extracts from the T-36 infected positive control. At 140 dai., the expression level of transclone number 4 had increased to comparable levels to the positive control and nine additional transclone plants assayed positive results but at lower severity, only detectable at a 1:20 dilution. At 189 dai., ELISA results indicated a reduction in the CTV antigen level in most of the plants and no virus could be found in some plants that had previously assayed positively (plants 13, 15, 19 and 31, Table 4.2). In addition, the CTV could only be detected in the T-36 infected control at 1:20 and

1:200 dilutions of extract. At 217 dai., only three transclones and the T-36 infected control showed detectable levels of CTV at 1:20 extract dilution. At 266 dai., virus could be detected only at a 1:5 dilution in transclone number 4, while the ELISA of the T-36 infected control indicated an increase in the replication level. Two transclones (numbers 10 and 17) remained negative for virus replication throughout the experiment. One of these plants died late in the experiment. Plant 56, the negative control, also died. However, observed symptoms (wilting) in both of these plants were not consistent with those caused by CTV. Therefore, we can not attribute their death to CTV infection. The decreased expression of the virus in both challenged transgenic clones and donor hosts may be attributed to several days of temperatures of 39 - 41°C caused by failure of the cooling system in the growthroom used, once during last days of December (at approximately 165 dai.) and a second time in the middle of March (at approximately 240 dai.). High temperatures are known to inhibit virus replication.

ELISA tests in experiment two (Figure 4.1B) indicated virus replication after 191 days of inoculation in two plants of the ten included in the experiment (Table 4.3). Virus was detected at a 1:5 dilution in the transgenic branch and a 1:20 dilution in the non-transgenic sour orange. This is more virus than could be detected in the inoculated non-transformed sour orange. The T-36 infected control showed the same behavior as in experiment one, a decrease in the detection of the virus at 119 and 147 dai., followed by an increase at 191 dai. All plants in experiment two were in the same growthroom as plants in experiment one; therefore they were also subjected to high temperatures. The dates of the sampling are the same as in experiment one although the dai. do not coincide because of the need to re-inoculate. In both experiments, ELISA results in non-inoculated transgenic sour orange plants used as controls (5/1/1 and 8/1/1) remained negative for the duration of the experiment. Thus we can attribute the positive ELISA readings obtained to virus infection.

Plants from experiment three (Figure 4.1C), were inoculated in the stem of the transgenic sour orange with isolate T-64. The inoculum tissue did not survive, even after several attempts to inoculate and several size and types of inoculum tissue were tried (bark and leaf pieces, and blind buds).

Elisa tests were also done on the sweet orange scions and the rough lemon rootstocks of transclones in experiment one (Figure 4.1) to determine whether the virus was moving throughout the plants. Table 4.4 shows that virus replicated in four of the scions in experiment one (plants 4,11,14 and 18) at comparable detection levels as in the transgenic sour orange interstock (Table 4.2). Table 4.5 shows that virus moved through the sweet orange scion, the transgenic sour orange interstock to the rough lemon rootstock in three plants, 4, 11 and 18. At 266 dai., virus could still be detected in plants 4, 14 and 18 in the scion and in plants 4, 11 and 18 in the rootstock.

Most of the transgenic sour orange clones containing the CTV-CP in experiment one allowed the replication of CTV strain T-36, although some of the transclones registered very low levels of virus replication and even no detectable levels of virus were found in some plants at certain moments in time. Plant number 4 in the first experiment seemed especially susceptible to virus infection, even more than the non-transformed negative control plant, and at one point it registered the same replication levels as the infected T-36 control. Graft inoculation is usually considered a severe form of challenge because the receptor plant is under continuous exposure to virus being generated in the inoculum tissue (Gonsalves and Garnsey 1989). Therefore, the donor tissue is often excised several weeks after grafting to reduce this effect. However, leaving the donor tissue on the test plant reduces the time required to evaluate the degree of protection. After 266 days, neither plant 4 or any other plant in the experiment showed any symptoms of CTV quick decline typically caused by the T-36 isolate in sour orange/sweet orange combinations. Only two plants out of ten in the second experiment showed detectable levels of CTV after 191 days of inoculation, and these levels were low. One of these plants originated from the 5/1/1 and the other from the 8/1/1 transgenic sour orange plants. Thus we could say that virus replicated in both 5/1/1 and 8/1/1 clones, although very few 8/1/1 clones were tested. The non-transformed inoculated controls showed slightly higher levels (Table 4.3). Neither branch of these plants (transformed sour orange and non-transformed sour orange) showed symptoms of CTV infection.

We have no doubt that further experiments should be done before we can conclude that the lack of symptom expression or the lack of viral replication seen in most of the transclones tested and the controls is correlated to the level of CP expression in the transgenic sour orange plants instead of being correlated to an ineffective inoculation procedure. It is important to indicate that horticultural and plant growth problems prevented us from making a more complete analysis and may also have affected the quantitatve assays done because it is known that titer varies according to tissue age and condition. We believe future experiments should include a larger number of both 5/1/1 and 8/1/1 transclones, more control plant numbers. To evaluate the effect of citrus transformation with the CTV-CP on decline, self rooted transgenic sour orange transclones will need to be topworked with sweet orange and tested in the natural combination that is disease sensitive.

TABLE 4.2. ELISA assay for replication of citrus tristeza virus (CTV) in transgenic sour orange clones in experiment 1 after challenge by graft inoculation.

		ELISA test[a] (days after inoculation)[b]					
Transgenic clone[c]	**GUS test[d]**	**63**	**100**	**140**	**189**	**217**	**266**
4	+	++	++	++++	++	++	+
9	+	-	-	++	++	-	-
10	+	-	-	-	-	-	dead
11	+	-	-	++	++	-	-
13	+	-	-	++	-	-	-
14	+	-	-	++	++	++	-
15	+	-	-	++	-	-	-
17	+	-	-	-	-	-	-
18	+	-	-	++	++	-	-
19	+	-	-	++	-	-	-
27	+	-	-	++	++	++	-
31	+	-	-	++	-	-	-
		Controls[e]					
56	-	-	++	+++	++	++	dead
M.V. sweet orange	-	-	-	-	-	-	-
5/1/1	+	-	-	-	-	-	-
Isolate T-36	-	++++	++++	++++	+++	++	+++

[a] ELISA = enzyme-linked immunosorbent assay. Replication of CTV-CP was determined by ELISA at OD $_{405nm}$ and recorded (-) negative and positive (+) with different severities (+, is detectable at 1:5 dilution; ++, is detectable at 1:20 dilution; +++, is detectable at 1:200 dilution;++++, is detectable at 1:2000 dilution). Samples were taken from the transgenic sour orange interstock.

[b] Transgenic sour orange clones (originated from 5/1/1 plant) were grafted onto rough lemon rootstock first, then grafted again, with Madam Vinous sweet orange so that the transgenic sour orange became an interstock for sweet orange. Plants were inoculated on 7/14/94 with rectangular bark and leaf pieces including midrib, from Madam Vinous T-36 infected donor plant.(Figure 4.1A).

[c] Plant identification number.

[d] GUS test = plants were assayed histochemically for β-glucuronidase activity (Jefferson 1987).

[e] Controls = 56, non-transformed sour orange used as interstock and challenged with T-36; M.V. sweet orange, non-inoculated control; 5/1/1, original transformed sour orange plant, not challenged; Isolate T-36, Madam Vinous sweet orange T-36 infected donor plant used as inoculum source and positive control.

TABLE 4.3. ELISA assay for replication of citrus tristeza virus (CTV) in transgenic sour orange clones in experiment 2 after challenge by graft inoculation.

			ELISA test[a] (days after inoculation)[b]							
			74 branch		119 branch		147 branch		191 branch	
Transgenic clone[c]	GUS test[d] T	NT	T	NT	T	NT	T	NT	T	NT
3	+	-	-	-	-	-	-	-	-	-
22	+	-	-	-	-	-	-	-	+	++
23	+	-	-	-	-	-	-	-	-	-
24	+	-	-	-	-	-	-	-	-	-
28	+	-	-	-	-	-	-	-	-	-
29	+	-	-	-	-	-	-	-	-	-
34	+	-	-	-	-	-	-	-	-	-
35	+	-	-	-	-	-	-	-	+	++
54	+	-	-	-	-	-	-	-	-	-
55	+	-	-	-	-	-	-	-	-	-
			Controls[e]							
B. sour orange	-		-		-		-		-	
8/1/1	+		-		-		-		-	
5/1/1	+		-		-		-		-	
Isolate T-36	-		++++		+++		++		+++	

[a] ELISA = enzyme-linked immunosorbent assay. Replication of CTV-CP was determined by ELISA at OD_{405nm} and recorded (-) negative and positive (+) with different severities (+, is detectable at 1:5 dilution; ++, is detectable at 1:20 dilution; +++, is detectable at 1:200 dilution;++++, is detectable at 1:2000 dilution). Samples were taken from the transgenic sour orange (branch T) and from the non-transgenic sour orange (branch NT). Plants were inoculated 10/2/94.

[b] Transgenic and non-transgenic sour orange clones were grafted onto rough lemon branches as separate scion branches. The non-transformed sour orange branch would be considered the negative control. Both branches were graft inoculated (Figure 4.1B).

[c] Plant identification number. Plant 3-29 originated from plant 5/1/1 and plants 34-55 from 8/1/1.

[d] GUS test = both branches transgenic (T) and non-transgenic (NT) sour orange were assayed histochemically for β-glucuronidase activity (Jefferson 1987).

[e] Controls = Brazilian sour orange, non-inoculated control; 5/1/1 and 8/1/1, original transformed sour orange plants, not challenged; Isolate T-36, Madam Vinous sweet orange T-36 infected donor plant used as inoculum source and positive control.

TABLE 4.4. ELISA assay for replication of citrus tristeza virus (CTV) in non-transformed Madam Vinous sweet orange scions in experiment 1 after challenge by graft inoculation.

	ELISA test[a] (days after inoculation)[b]					
Transgenic clone[c]	**63**	**100**	**140**	**189**	**217**	**266**
4	NT[d]	++	++++	++	+	+
9	NT	-	-	-	-	-
10	NT	-	-	-	-	dead
11	NT	-	++	++	+	-
13	NT	-	-	-	-	-
14	NT	+	++	++	+	+
15	NT	-	-	-	-	-
17	NT	-	-	-	-	-
18	NT	+	+++	++	+	+
19	NT	-	-	-	-	-
27	NT	-	-	-	-	-
31	NT	-	-	-	-	-
	Controls[e]					
56	-	++	+++	++	++	dead
M.V. sweet orange	-	-	-	-	-	-
5/1/1	-	-	-	-	-	-
Isolate T-36	++++	++++	++++	+++	++	+++

[a] ELISA = enzyme-linked immunosorbent assay. Replication of CTV-CP was determined by ELISA at OD $_{405nm}$ and recorded (-) negative and positive (+) with different severities (+, is detectable at 1:5 dilution; ++, is detectable at 1:20 dilution; +++, is detectable at 1:200 dilution;++++, is detectable at 1:2000 dilution). Samples were taken from Madam Vinous sweet orange scions.

[b] See Figure 4.1A.

[c] Plant identification number.

[d] NT= not tested

[e] Controls = 56, non-transformed sour orange used as interstock and challenged with T-36; M.V. sweet orange, non-inoculated control; 5/1/1, original transformed sour orange plant, not challenged; Isolate T-36, Madam Vinous sweet orange T-36 infected donor plant used as inoculum source and positive control.

TABLE 4.5. ELISA assay for replication of citrus tristeza virus (CTV) in non-transformed rough lemon rootstock in experiment 1 after challenge by graft inoculation.

	ELISA test[a] (days after inoculation)[b]					
Transgenic clone[c]	**63**	**100**	**140**	**189**	**217**	**266**
4	NT[d]	-	+++	++	+	+
9	NT	-	-	-	-	-
10	NT	-	-	-	-	dead
11	NT	-	++	++	+	+
13	NT	-	-	-	-	-
14	NT	-	-	-	-	-
15	NT	-	-	-	-	-
17	NT	-	-	-	-	-
18	NT	-	++	++	+	+
19	NT	-	-	-	-	-
27	NT	-	-	-	-	-
31	NT	-	-	-	-	-
		Controls[e]				
56	-	++	+++	++	++	dead
M.V. sweet orange	-	-	-	-	-	-
5/1/1	-	-	-	-	-	-
Isolate T-36	++++	++++	++++	+++	++	+++

[a] ELISA = enzyme-linked immunosorbent assay. Replication of CTV-CP was determined by ELISA at OD $_{405nm}$ and recorded (-) negative and positive (+) with different severities (+, is detectable at 1:5 dilution; ++, is detectable at 1:20 dilution; +++, is detectable at 1:200 dilution;++++, is detectable at 1:2000 dilution). Samples were taken from rough lemon rootstock.

[b] See Figure 4.1A.

[c] Plant identification number.

[d] NT = not tested

[f] Controls = 56, non-transformed sour orange used as interstock and challenged with T-36; M.V. sweet orange, non-inoculated control; 5/1/1, original transformed sour orange plant, not challenged; Isolate T-36, Madam Vinous sweet orange T-36 infected donor plant used as inoculum source and positive control.

CHAPTER 5
SUMMARY AND CONCLUSIONS

Different factors that affect the frequency of transformation were evaluated and transformed citrus plants containing and expressing the citrus tristeza virus coat protein gene were obtained.

Genetic Transformation of Citrus Plants

Transformation efficiency was increased due to the use of a different vector plasmid (pGA482GG) and in Key lime due to the use of a different *Agrobacterium* strain. Two types of explants were tested: internodal stem segments and node segments. Stem segments were more readily transformed than node segments in all four cultivars evaluated. If we consider that *Agrobacterium* typically invades wounded plant cells in order to insert foreign genes into the plant genome, thus rendering preformed meristematic cells a poor target for this method of transformation, it seems reasonable to have obtained low frequencies of transformation when using nodal segments. Likewise, the higher levels of transformation seen in stem segments may be related to the organogenic events taking place while new adventitious shoots are being formed.

The use of the selectable-marker NPTII was not very effective for the selection of transformed plantlets, as many escape shoots regenerated frequently. Probably the use of a different selectable-marker should be considered. The reporter gene GUS proved helpful for identification of transformed tissues, whether chimeric or entirely GUS+. However, many of the putatively transgenic shoots obtained appeared chimeric for GUS expression (330 shoots in total, data not shown). Some of these were stained in only a very tiny sector of the basal sections assayed (approximately 240 out of the 330 shoots), while in others much larger sectors stained (90 shoots in total). In the former instance PCR failed to amplify the expected fragment, i.e. in only one Key lime shoot where a very small GUS+ sector was observed was the CTV-CP corresponding fragment amplified (lane 7 of Figure 3.3). In the latter instance, amplification was observed in a few more cases; in three chimeric shoot samples shown in Figure 3.2 and four chimeric shoot samples shown in Figure 3.3. Moreover, when the stained section was a small sector of the basal sections assayed, PCR amplification would, most of the time, yield a product when the test was done at a very early stage of shoot growth (when the shoots were one to two centimeters in length), but in many cases, after a year or so of growth in the greenhouse, subsequent assays did not detect GUS activity and a second PCR amplification would fail to amplify the product. This is the case of the plant in lane 7 of Figure 3.3 which is a Key lime plant regenerated from a node that was GUS+ and CTV-CP PCR positive the first time tested. It seemed that the putatively transgenic shoot outgrew the chimeric sector previously detected and neither the reporter gene GUS nor the NPTII, GUS and CTV-CP sequences could be detected again. Explanations for such variable expression of the GUS gene include chimerism of the regenerated plants, difficulty of penetration of the X-glucuronide substrate, post-insertional modification of the introduced gene such as methylation, post-translational modification of β-glucuronidase, and variation of the points of gene insertion among the transclones (Serres *et. al* 1992). Thin sectioning revealed that penetration of the substrate was not the major reason for variable GUS expression. We did detect in some transgenic shoots visual evidence, such as sectoring expression, for the presence of chimeric regenerates.

Our results indicate that more than one test should be used to characterize plants that have gone through the transformation process. GUS assays can commence when plants are in tissue culture. Plants that test positive in tissue culture should also be tested after they are established in the greenhouse. These tests should include a repeat of GUS and PCR assays. However, we think that, for the most part, PCR amplification of specific DNA fragments proved very effective for identification of transgenic plants that most likely had incorporated a foreign gene in their genomes. Routine use of PCR would reduce the number of plants to be tested by Protein blot or DNA blot analyses which are more laborious.

Rooting and adaptation to soil of transgenic plants remains a bottleneck. Of the 380 putatively transgenic plants (GUS+) obtained in all of the experiments only 40 successfully rooted and survived the adaptation stage (data not shown). Size of the transgenic shoots at the time of transfer to soil seems to have a major influence and efforts to obtain more vigorous shoots should be made. Absence of BA in the medium and placing the stem segments horizontally on the medium induced regeneration of fewer shoots, but the shoots were larger and rooted more frequently. Micrografting of transgenic shoots was attempted with shoots regenerated from experiments with the strain A518, but the grafted shoots developed tumors in the budunion and eventually died. However, we believe that micrografting might be a good alternative for circumventing the rooting problem when using an engineered *Agrobacterium* strain. Differences among varieties of citrus may also have an effect on the success of surviving in soil since all of the transgenic Key lime plants obtained were much smaller and thiner than shoots of other species.

The use of particle bombardment followed by *Agrobacterium* inoculation as a wounding mechanism to enhance transformation was not effective. Moreover, regenerated transformed shoots were bleached and fragile. However, only Key lime was used in these experiments.

The presence of the gene for CTV-CP, inserted into the host genome by the vector, was confirmed by DNA blot analysis. Plants identified as transgenic by GUS assays and verified by PCR and DNA analysis were tested for CTV-coat protein expression by immunoblot analysis. Samples were incubated with either polyclonal antibody 894 or monoclonal antibody MCA-13. Protein blot analyses detected CTV protein expression in plants where original sections either stained entirely for GUS activity or where a large sector of the assayed cross-section stained. No protein could be detected in chimeric transgenic plants where only a tiny sector of the cross-section stained during GUS assays.

Challenge Inoculation of Transformed Citrus Plants

CP-mediated protection was evaluated by detecting the replication or the lack of replication of CTV isolate T-36 in challenged plants. Two transgenic sour orange lines obtained from the transformation experiments were used for the challenging experiments. Sour orange was the most popular rootstock used for sweet orange production in almost every country until the arrival of CTV, after which it had to be replaced because of its susceptibility to the disease.

Nor or very low detection of the CP produced by transgenic plants was observed when using ELISA assays, and only when using certain monoclonal and polyclonal antibody combinations. Whereas detection of viral CP in T-36 CTV infected control plants was clearly demonstrated in every combination. We believe that the detection or lack of detection of the CP in transgenic plants may be due to one or more reasons, serological and biological. The CP gene transferred to the transgenic plants may have been somehow rearranged such that the availability of different epitopes has changed. Another possibility is that some changes may have been induced during tissue culture regeneration of citrus plantlets. It may be due to the different specific recognition sites within the CP of the monoclonal antibodies. The fact that MAB 3DF1 could detect the CP in transgenic plants in ELISA assays may be reasonable if we consider that, since the most critical amino acid for reactivity is at position 2 of the CP (Pappu *et al.* 1994, 1995), the extreme location of this epitope may allow it to stick out, making it available for detection even if the protein is not denatured. The results may also depend on how the crude extracts were prepared, whether proteins in the samples were denatured versus nondenatured as in Protein blots and ELISA assays, respectively. Depending upon protein conformation, different epitopes critical for detection with different MABs may be or may not be available for exposure, causing various results in serological detection. However, futher analyses need to be done to evaluate these considerations.

Most of the transgenic sour orange clones containing the CTV-CP in experiment one allowed the replication of CTV strain T-36, although some of the transclones registered very low levels of virus replication and even no detectable levels of virus were found at certain times. Plant number four seemed especially susceptible to virus infection, even more than the non-transformed negative control plant (at one point it registered the same expression levels as the infected T-36 control). Graft inoculation is usually considered a severe form of challenge because the receptor plant is under continuous exposure to virus being generated in the inoculum tissue (Gonsalves and Garnsey 1989). In these experiments, we provided a permanent source of the severe isolate T-36 which provided a stronger challenge pressure than happens under natural conditions.

After 266 days, neither plant number four nor any other plant in experiment one showed any symptoms of CTV quick decline typically caused by the T-36 isolate in sour orange/sweet orange combinations. Only two plants out of ten in the second experiment showed detectable levels of CTV after 191 days of inoculation, although at low levels. The non-transformed inoculated controls showed slightly higher levels. One of these plants originated from the 5/1/1 and the other from the 8/1/1 transgenic sour orange plants. Thus we could say that virus replicated in both 5/1/1 and 8/1/1 clones, although very few 8/1/1 clones were tested. Neither of these plants showed symptoms of CTV infection.

ELISA results done on the sweet orange scions and the rough lemon rootstocks in experiment one determined that the virus was replicating in parts of the plants other than the transgenic sour orange interstock and that it was moving through the transgenic interstock down to the non-transgenic rootstock at least in three plants (4,11 and18). However some interesting discrepancies in virus replication between rootstock, interstock and scion remain unexplained and more experiments should be made before any conclusions about virus movement are made.

We could not consistently infect the transclone plants with CTV even after several inoculations. This was the case not only in experiment three but also in the first and second experiments, where only one negative control was successfully inoculated or some transclone plants where the graft had died were removed from the experiments. This shows the difficulty of doing these kinds of tests in a system where the virus can not be easily inoculated into transgenic plants and where a considerable amount of time must pass before one can determine whether inoculation has been successful. Also we could not compare our results to the high levels of resistance reported in similar challenging reports in other species for several reasons, a major one being that they used either aphid inoculation or plant sap inoculation which do not represent a permanent source of inoculum, and second reason being that most of the species are annuals.

We have no doubt that further experiments should be done before we can conclude that the lack of symptom expression or the lack of viral replication seen in most of the transclones tested and the controls is correlated to the level of CP expression in the transgenic sour orange plants instead of being correlated to an ineffective inoculation procedure. It is important to indicate that horticultural and plant growth problems prevented us from making a more complete analysis and may also have affected the quantitatve assays done because it is known that titer varies according to tissue age and condition. We believe future experiments should include a larger number of both 5/1/1 and 8/1/1 transclones and hopefully other transclones as well as higher control plant numbers. However, it may be valuable to evaluate sweet orange grafted onto self-rooted sour orange transclones inoculated with CTV as a first screen. This was not possible in the present experiment due to the length of time necessary to propagate a sufficient number of self-rooted transgenic sour orange plants.

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BIOGRAPHICAL SKETCH

María Alejandra Gutiérrez Espinosa was born on June 28, 1960, in Mérida, Yucatán, México. Alejandra attended the Universidad Autonoma Metropolitana in México City, between 1979 and 1983, where she earned a Bachelor of Science degree of Ingeniero Agrónomo with a major in crop production. During 1983 to 1985, she worked as a research assistant at the Comision Nacional de Fruticultura, México City where she worked mainly in micropropagation for commercial purposes of tropical fruits and ornamentals. From 1986 to 1987, Alejandra completed her Master of Science degree in fruit crops at the Colegio de Postgraduados, Montecillo, México. Starting in 1988, Alejandra was appointed research associate in the Department of Fruit Crop Science in the same institution. Beginning in 1990 Alejandra initiated studies at the University of Florida to pursue a doctoral degree in horticultural science. Upon completion of her studies, Alejandra will rejoin the Department of Fruit Crop Science at the Colegio de Postgraduados in Mexico.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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